Identification, Chromatographic Estimation and Safety assessment of some Selected Extractable and Leachables in Liquid Pharmaceutical Formulations

A Thesis submitted to Gujarat Technological University for the Award of

Doctor of Philosophy

in

Pharmacy

by

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under supervision of
Prof (Dr.) Navinchandra Sheth

Co-supervised by
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GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

August- 2025

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11.8.25

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ABSTRACT

Introduction

Extractables and Leachables are types of contamination and may present in ultimate pharmaceutical products. Extractables and Leachables both are organic and inorganic chemical compound extractables are released from the surface during laboratory conditions while leachables are travel from the surface during life of product with the function of time and temperature. For example, plasticizers are the materials which leach from the plastic materials. As plastic is a widely use material for packaging in pharmaceutical industries but it has a disadvantage of leaching and can influence the safety, efficacy and quality of the product. Extractables and leachables are entre into the product through various routes like primary packaging materials, single use system components, residual cleaning agents, antistatic agents, secondary packaging materials, inks and dyes, antioxidants and stabilizers, lubricants, emulsifiers, molding agents, vulcanizing agents, residual monomers, phthalates, polymers and monomers, nitrosamine and polyaromatic hydrocarbons. Many modern analytical techniques like GC-MS/MS, LC-MS/MS and ICP-MS have been employed based on their chemical nature.

Objectives

The main objectives of this study are to identify the most prevailing extractables and leachables. Also, to develop and validate gas chromatography- mass spectroscopic methods for the identification of extractables and leachables and to perform the invitro toxicity study for the selected extractables and leachables.

Materials and Methods

Selected extractables and leachables standards namely naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, alpha methyl styrene, diethyl azelate and tributyrin was procured from sigma Aldrich. HPLC grade solvents were procured loba chem, rankem, India. Millipore water system was used for water. Sample preparation involved stock solution of selected substances by using acetone. Analytes were separated on column SH-Rxi-5 Sil MS (30.0m, 0.25mm, 0.25mcm) with the use of helium as a carrier gas and injection volume of 10mcL. The method was validated according to ICH Q2(R1) guidelines and all the data are falls within the acceptance criteria. marketed pharmaceutical formulations were procured from the local market to carry out the application of the method. For the toxicity study HEK-293 and MCF-7 cell line were procured from Pune, Maharashtra.

Results and Discussion

Estimation of selected extractables and leachables were carried out by GC-MS. The detection was performed by recording a mass of naphthalene was 128, 127 and 129; mass of acenaphthene was 153, 154 and 152; mass of anthracene was 178, 176 and 76; mass of fluoranthene was 202, 200 and 101; mass of pyrene was 202, 200 and 101; mass of alpha methyl styrene was 118, 117 and 103; mass of diethyl azelate was 83, 55 and 152; mass of tributyrin was 72, 44 and 43. Both the method was found linear as per the value of R2 for both the group was found to be $\geq$ 0.990 from the range 30µg/mL to 10µg/mL for group 1 and for group 2 alpha-methyl styrene, diethyl azelate and tributyrin it was found from 250-50 ng/mL, 250-50 ng/mL and 50-10 ng/mL respectively. The retention time for group 1 i.e., naphthalene, acenaphthene, anthracene, fluoranthene and pyrene it was found to be 14.34±0.2 minutes, 21.41±0.2 minutes, 27.72±0.2 minutes, 32.43±0.2 minutes, 33.19±0.2 minutes respectively and

for group 2 i.e., alpha methyl styrene, diethyl azelate and tributyrin it was found to be 5.72±0.2 minutes, 23.77±0.2 minutes and 26.69±0.2 minutes respectively. The value of % recovery was found to be 98-102% i.e., ±2% of 100% for all standards also the developed method was within the range of %CV i.e., ≤2%. Other parameters like robustness were performed using various parameters like change in flow rate, temperature and hold time and pressure was found to be within the range. Specificity data for both the group was found to be specific for analytes only. A toxicity study of the selected analytes was performed on the cell line. All the analytes were applied on the marketed pharmaceutical formulations.

Conclusion

Simultaneous estimation of naphthalene, acenaphthene, anthracene, fluoranthene and pyrene was done as a set of analytes and another method was established of alpha methyl styrene, diethyl azelate and tributyrin. The present work was successfully applied for the detection of extractables and leachables in pharmaceutical formulations available in the local market.

Key words:

Extractables, Leachables, Pharmaceutical Liquid Formulations, GC-MS, ICH Q2(R1) Guideline, Cell Line.

Acknowledgement and Dedication

Methodology should not be a fixed track to a fixed destination but a conversation about everything that could be made of happens. Research is creating new knowledge. The value of information provided by such self-driven people is always of high importance. At present occasion, when I am ready to accept the challenges in the field of pharmaceutical research, I sense that the present exercise for investigation would not have touched objectives without blessing and guidance from dedicated researchers and well-wishers.

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*Thankful to all ever remain.....****Stuti V. Pandya***

Table of Contents

List of Abbreviations

Abbreviation	Full Name
AR	Analytical Reagent
MS	Mass Spectrometry
EI	Electron Impact
MS/MS	Tandem Mass Spectrometry
HPLC	High Performance Thin Layer Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography- Tandem Mass Spectrometry
GC-MS	Gas Chromatography- Mass Spectrometry
IR	Infrared Spectroscopy
FT-IR	Fourier Transformer- Infrared Spectroscopy
m/z	mass-to-charge ratio
CV	Co-efficient of Variance
RSD	Relative Standard Deviation
CC	Calibration Curve
RT	Retention Time
STD	Standard
SD	Standard Deviation
SS	Stock Solution
LOD	Limit of Detection
LOQ	Limit of Quantification
Avg.	Average
E&L	Extractables and Leachables
IP	Indian Pharmacopoeia
BP	British Pharmacopoeia
USP	United States Pharmacopoeia
ICH	International Council on Harmonization

USFDA	United States Food and Drug Administration
EMEA	Europe Middle East and Africa
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
PDA	Parenteral Drug Association
MTT	(3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
DMSO	Dimethyl Sulfoxide
MP	Melting Point
BP	Boiling Point
HCl	Hydrochloric Acid
Vol.	Volume
NS	Normal Saline
SWFI	Sterile Water for Injection
CFR	Code of Federal Regulations
IUPAC	International Union of Pure and Applied Chemistry
NIST	National Institute of Standards and Technology
DCM	Dichloro Methane
IPA	Isopropyl Alcohol
Hrs.	Hours
HEK	Human Embryotic Kidney
NCCS	National Centre for Cell Science
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
IC	Inhibitory Concentration
OINDP	Orally Inhaled Nasal Drug Product
PODP	Parenteral and Ophthalmic Drug Product
PQRI	Product Quality Research Institute

List of Symbols

Symbol	Full Name
%	Percentage
>	More than
<	Less than
≥	Greater than or Equal to
≤	Less than or Equal to
=	Is Equal to
+	Addition
×	Multiplication
α	Alpha
β	Beta
µg	Microgram(s)
µl	Microliter(s)
i.e.	That is
L	Liter
M	Molar
mg	Milligram(s)
ng	Nanogram
Min	Minute
ml	Milliliter(s)
nm	Nanomiter
R^2	Correlation co-efficient
Temp.	Temperature
Sr. No.	Serial number
v/v	Volume by Volume
w/w	Weight by Weight
Conc.	Concentration

PPM	Parts per million
°C	Degree Celsius
&	And
±	Plus, or Minus
kPa	Kilopascal
cm	Centimeter
sec	Second
Hrs.	Hours
/	Per
pH	Potential of Hydrogen
CO_2	Carbon Dioxide
MW	Molecular Weight

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Chapter- 1

Introduction

1.1 Impurity

1.1.1 Impurity (IP 2022, vol.1, page no. 1175)

The definition of impurity is any ingredient that is not the chemical entity that characterizes A medication product, or a drug ingredient used in pharmaceuticals, or the excipient in this is not a drug product. It comprises, the substance of the drug, among other things, degradation products might occur during storage, as well as those that apply to dosage forms might similarly form all through manufacturing and loading. Contaminants may be of two types

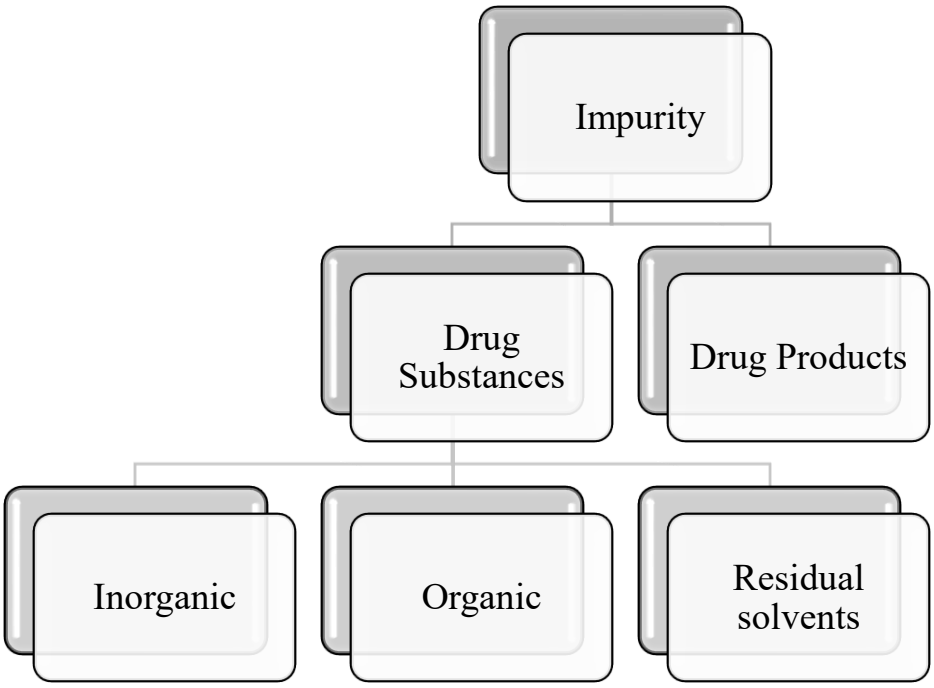


Fig 1.1: Classification of Impurities as per IP

1.1.1.1 Impurities in Drug Substances

The impurity test for the drug products is modified to include degradation products and is based on the test for the active ingredient in the monograph.

- **Inorganic impurities**, which include ligands, chemicals, catalysts, heavy-additional metals, mineral salts, etc., are usually the product of manufacturing operations. Raw materials used as inputs and storage conditions have an impact on impurities. There should be no issues with the detection and measurement of such contaminants using traditional physicochemical techniques.
- **Organic impurities** can be related to drugs or processes and include identifiable, unidentified, or completely unknown impurities.
- During the production of a pharmacological ingredient, solvents which can be either organic or inorganic liquids are utilized as carriers to prepare suspensions or solutions. These are therefore typically known to be harmful and can be managed within appropriate bounds. Additionally, some medications require limitations for specific solvents where deviation in levels needs to be controlled, together with a broad solvent limit that is still present in the final pharmacological components.

1.1.1.2 Impurities in Drug Products

Dilapidation products include the dynamic ingredient's degradation products in the medication product, the active ingredient's reaction products with the immediate the active component, the container and closure system's reaction products with the excipient, and the goods of interactions amid the different drugs in an amalgamation product.

The approval criteria consider both known and unknown degradation products.

Stability tests, analysis of regular production batches, and forced degradation experiments are used to identify those contaminants. For contaminants that appear during the dosage form's manufacturing or storage, either broader limits or both, more controls can be needed.

1.1.2 Impurity: (BP 2009, vol. 1, page no. 30)

According to BP the material originates to have an contamination not measurable through means of the set trials is none of pharmacopeial excellence so, if the countryside or volume of the contamination found is discordant by way of good pharmaceutical practice.

In that kind of trials, the total amount of impurities is given in afterthoughts both as a parts per million by weight (ppm) or percentage (%).

For Chromatographic tests

- Percentage

For other tests:

- Parts per million (up to 500ppm)

Fig 1.2: Units of Impurities as per BP

The chromatogram of the test sample is equated with the chromatogram of the reference sample in that the comparison of spot or peak can be check in case of presence of spot or peak except the known material, that may include in impurity and that spot or peak are known as a secondary spot or peak.

1.1.3 Impurity (USP, page no. 2510)

If new definitions of purity and impurity may be applied to a material that was previously thought to be pure. The types of impurities are as per the following,

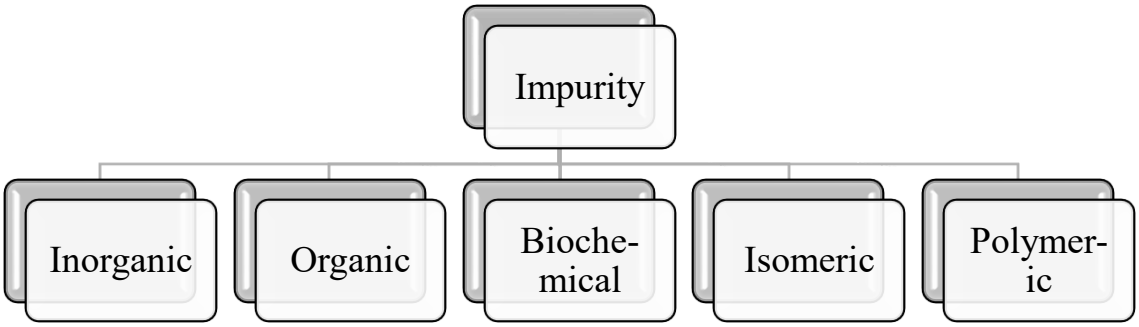


Fig: 1.3 Classification of Impurities as per USP

Definitions:

Foreign Substances

- This are introduces by contamination or adultration

Toxic Impurity

- The impurity has undesirable biological activity and it require quantification and identification test

Concomitant Components

- These are available in bulk products and are not considered to be impurity

Single Impurity

- It includes process related or degradation product related impurity

Ordinary Impurity

- It has no any undesirable biological activity

Fig 1.4: Types of Impurities as per USP

1.1.4 Impurity (ICH): (ICH guideline)

The International Council for Harmonization (ICH) is the joint organization of the regulatory authorities of United States, Europe and Japan for the technical requirements of drugs intended for human consumption.

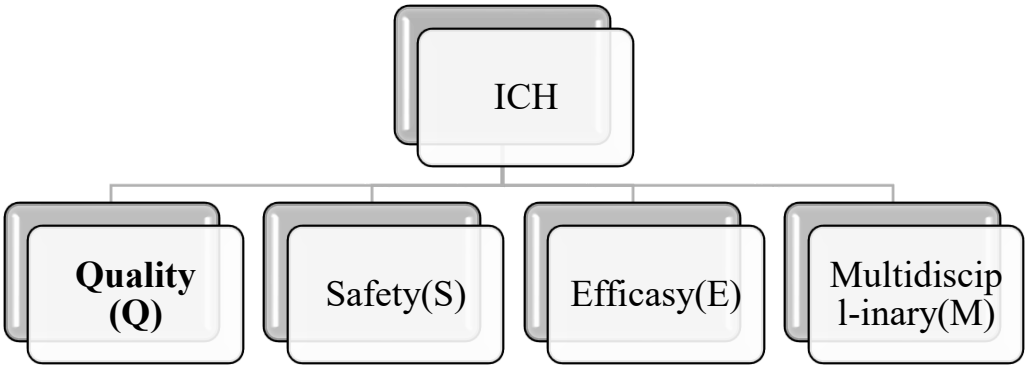
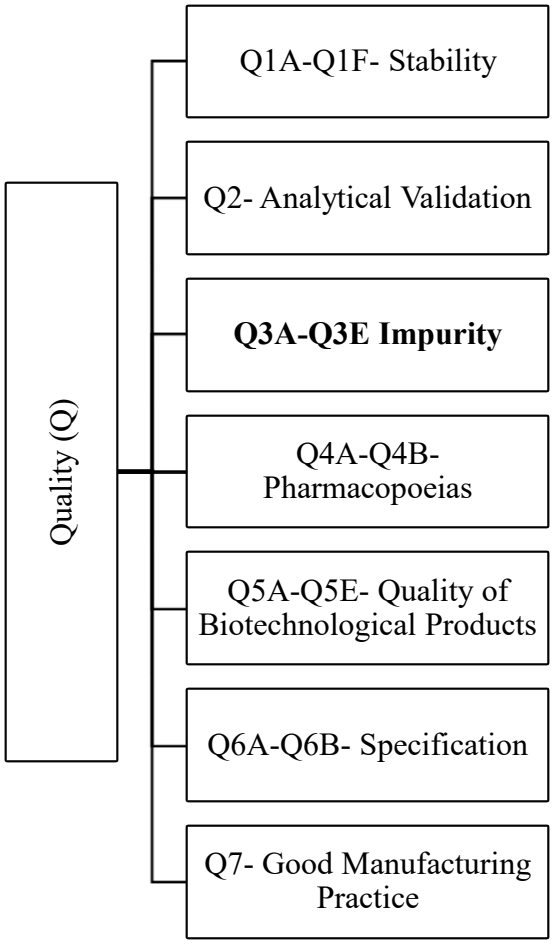


Fig 1.5: Classification of ICH guidelines

- **ICH Q (Quality) Guideline**



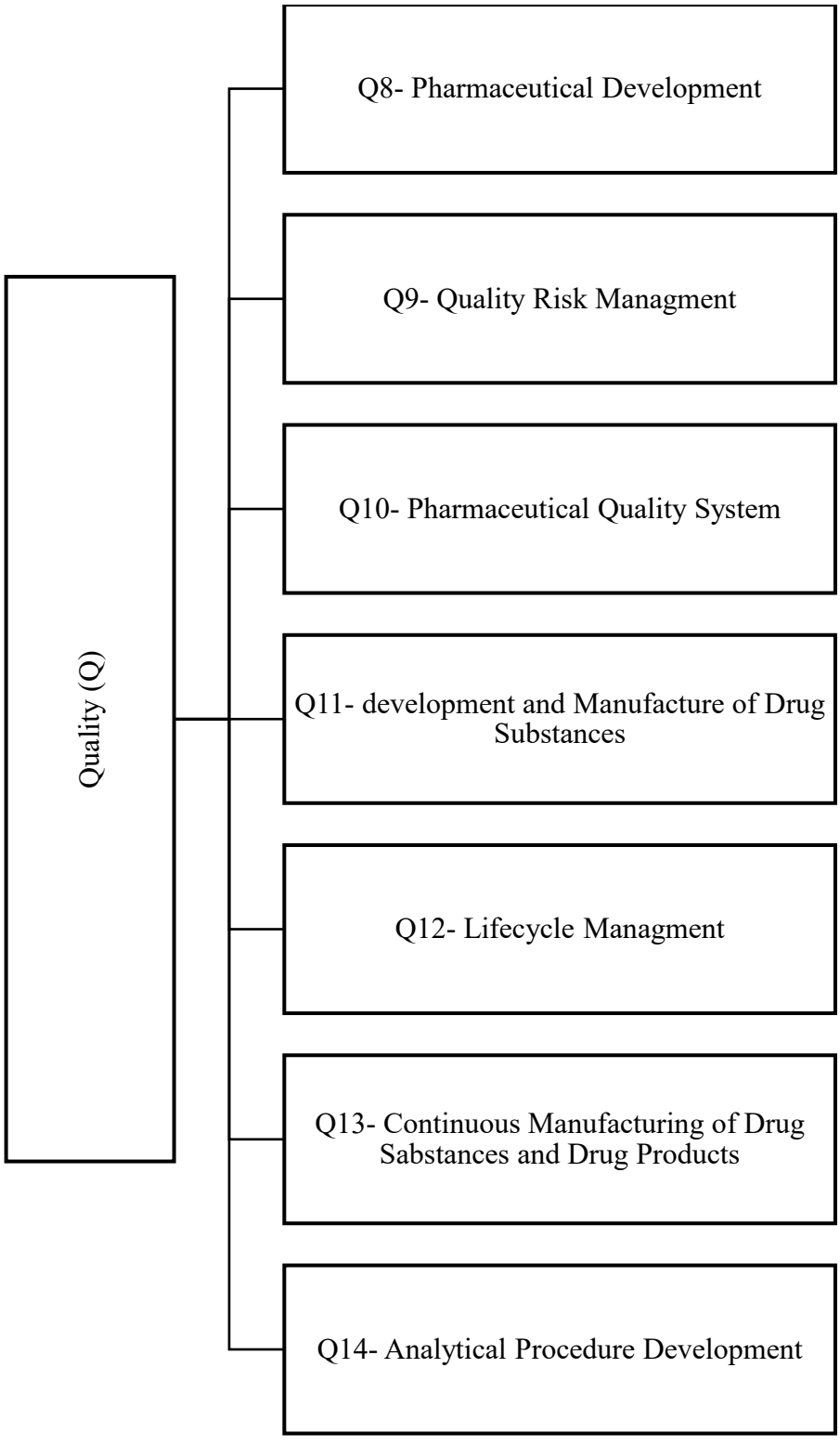


Fig 1.6: Classification of ICH Q guideline

- **ICH Q3A-Q3E Impurity:**

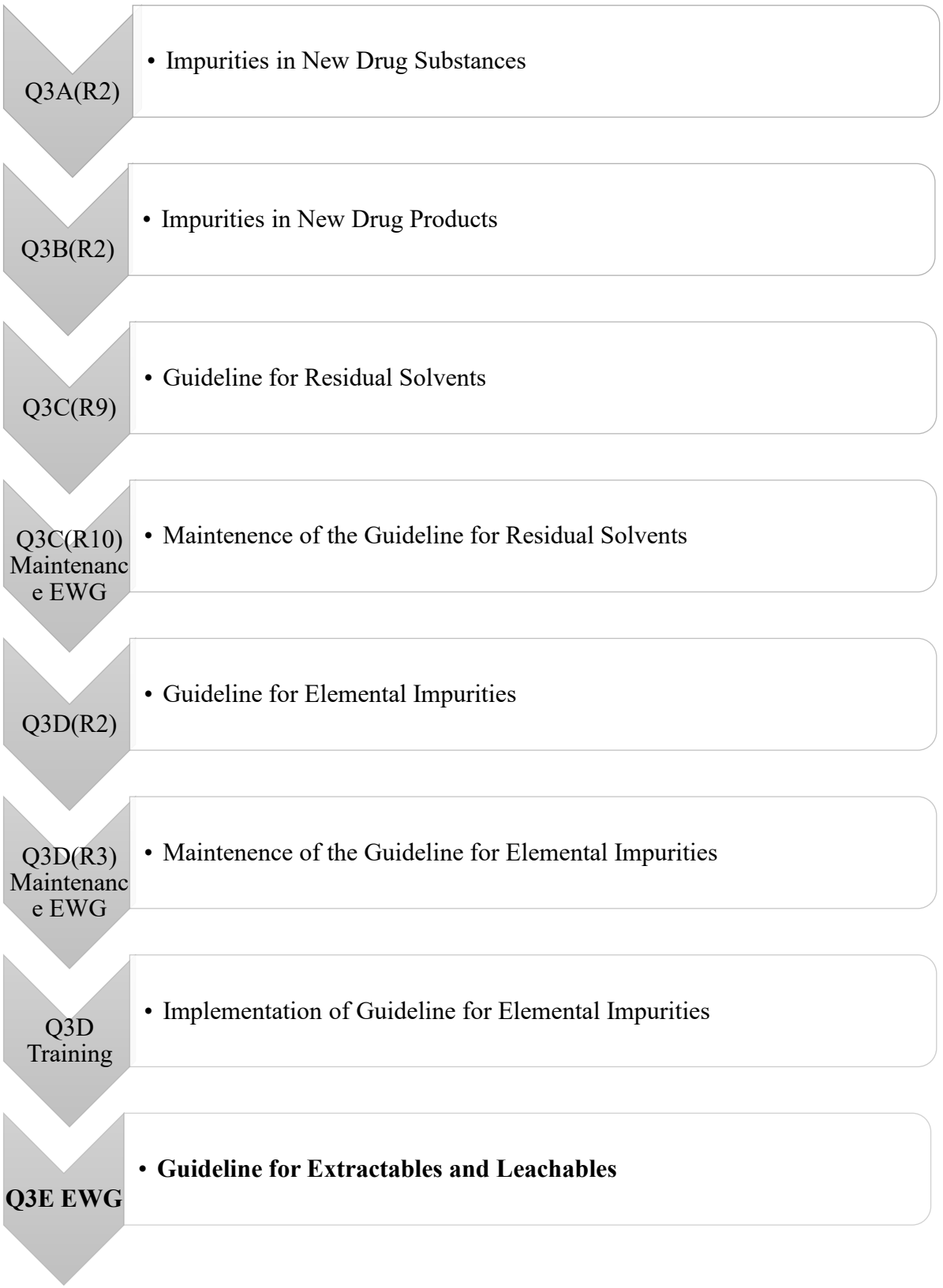


Fig 1.7: Classification of ICH Q3 Guideline

1.2 Impurity Profiling:

The name Impurity profiling is of a set of analytical events, the objective of which is the identification/structure elucidation, exposure and quantitative determination of both inorganic and organic impurities along with residual solvents in pharmaceutical formulations and in addition to bulk drugs.

1.3 Extractables and Leachables: (Jenke, D et. al.,)

1.3.1 Extractables:

Extractables are any chemical substances that, in the "worst-case scenario" of possible impurities, can be "drawn out" of a manufacturing component or packaging material under extreme circumstances like high temperatures or powerful solvents. extractables are substances that are taken out of a container closure system using strong solvents. To find out what compounds a patient might be exposed to, extractables studies are conducted. A substance is evaluated using extractables experiments. The material like extractables can be extracted with suitable solvents for example water, hexane, isopropanol, methyl chloride and so on. Extractables are defined as organic and inorganic chemical classes that, depending on temperature and time, can be liberated from the surfaces of parts used in the production and storage of pharmaceutical goods in a laboratory setting. Also, extractables are resources of the constituents of container/closure system, drug product delivery system and manufacturing system. Examples of extractables are slip agents, processing aids, surfactants, lubricants, degradants, and plasticizers.

1.3.2 Leachables:

Leachables are the subclass of those compounds that can actually travel into a drug product under usual storage conditions, meaning they leach out from the packaging into

the drug formulation through straight contact, fundamentally, leachables are a subset of extractables that are released under characteristic usage circumstances. Leachables studies are performed to determine the chemicals to which patients are exposed. Leachables investigations are evaluations of the pharmaceutical product. Therefore, the leachables are characteristically a division of extractables but it is not possible always. Leachables are defined as chemical substances, both organic and inorganic, that move from system components into a pharmaceutical product during the system's lifetime. Thus, extractables are significant because they help to categorize potential leachables. The resources of leachables are container closure system, syringe barrels and plunger in prefilled syringe, rubber stoppers and glass vials, nasal pumps, valve components for pressurized meter dose inhalers, bottles and caps. Drug product and delivery systems- IV bags, drug delivery pumps and mouthpieces, administration sets. Secondary packaging materials like fill pouches for low density polyethylene ampules, ink on label. In order to identify and measure the possible pollutants, both extractables and leachables are usually examined using advanced analytical methods like chromatography in conjunction with mass spectrometry. Common examples of leachables are polymer degradation products, ink and adhesives from labels, residual manufacturing solvents, antioxidants, dyes, metal catalysts etc.

Fig 1.8: Various Packaging Materials

1.3.3 Sources of Extractables and Leachables

The sources of extractables and leachables could leak into the drug product when exposed to the drug formulation during use and storage; this can also include production process elements that come into contact with the drug product, such as processing equipment, tubing, and filters. Determining the safety risk to patients requires assessing the possible toxicity of identified leachables. E&L impurities can originate from a variety of possible sources due to the extremely intricate procedures used in the production of drug delivery systems, implantable medical devices, packaging materials, and bioprocess materials. These include production components like bags, tubing, and filters, as well as packaging materials including vials, foils, inks, and coatings. Another risk factor is that a large number of manufacturing components used today are composed of elastomeric and single-use plastic materials, whose properties can greatly raise the possibility of extractable exposure or create "a much greater chance of having... leachables with direct health risks." A subclass of impurities known as extractables and leachables is produced when pharmaceutical goods interact with their

production components, drug delivery systems, and packaging. Plasticizers, antioxidants, and polycyclic aromatic hydrocarbons (PAHs) are examples of common E&Ls that can move from a variety of sources into a dosage form.

1.3.3.1 Primary packaging materials:

The packaging materials which are in direct interaction with the product or the primary layer around the product are known as primary packaging material. This type of materials is very prone to the pharmaceutical product as they have direct contact with the product. Selection of the primary packaging material should be done with high care, and the material should have lowest reactivity with the drug product. Examples of the primary packaging materials are containers, closures (container closure system), ampoules, vials, dosing dropper, syringe, strip packaging, blister packaging (polyvinyl chloride blister or alu-alu blister).

1.3.3.2 Single use system components:

The resources which may not be cleaned after it is used or that are disposed of once it is used. Single-use parts are made to be thrown away after only one use. It is not essential to prepare items before using many disposable systems because they are delivered to the user already cleaned and sterilized. As a result, the customer site no longer requires cleaning and sterilization procedures. Some government bodies have started banning the use of single use components so far. Examples of the single use system components are filters, process containers (bags), Tubing, Connectors, Gaskets, Valves, Residual cleaning agent, Antistatic agent.

1.3.3.3 Secondary packaging material:

The materials which are not in direct contact with the product or just next to the primary

packaging materials are known as secondary packaging material. The materials used to bundle main packaging or group products together are known as secondary packaging materials. They may consist of shrink wrap, paperboard, cardboard, and plastic. Products should be protected by secondary packaging that is both durable and easily opened to allow personnel to access the product without causing damage. Examples of secondary packaging materials are cardboards cartons, cardboard boxes, cardboard or plastic crates, paper, corrugated fibers.

1.3.3.4 Inks & dyes:

These materials are used for labeling onto the packaging material and glue is used to-

stick label on the product. Inks and dyes are regarded as possible sources of pollutants in the context of extractables and leachables, which can spread from packaging materials like labels and cartons into a product. Particularly if the product and the packing material come into direct touch or if the ink is not made specifically for pharmaceutical use, the pigments and additives found in inks and dyes may seep into the product. Examples of materials used as inks and dyes are adhesives, glue, also, the leaching capability of the ink can be greatly influenced by the kind of pigments, solvents, and additions employed in it.

1.3.3.5 Antioxidants & stabilizers:

Antioxidants are the material which is meant to avoid the oxidation of excipients and active substances in the final product. Stabilizers are used to inhibit deterioration, contamination, spoilage by bacteria and fungi. Examples of antioxidants and stabilizer types of materials are butylated hydroxyanisole (BHA), ascorbic acid, butylated

hydroxytoluene (BHT), alpha tocopherol (Vit-E). alginate, agar, gelatin, guar gum, Starch, xanthan gum.

1.3.3.6 Lubricants, emulsifiers & molding agent:

Lubricants are proposed to diminish friction. The substances which are used to stabilize the emulsion are known as emulsifiers. By keeping liquids that do not often mix from separating, an emulsifier stabilizes emulsions. Molding agents are used to avert sticking the materials outward. In order to facilitate processing, such types of components are frequently added to other components during manufacturing. However, if they are not carefully chosen and managed, they may leak into the drug product. Examples of lubricants, emulsifiers and molding agents are mineral oil, petroleum oil, vegetable oil, petroleum jellies, graphite, molybdenum disulphide. emulsifying wax, polysorbate 20, cetearth 20.

1.3.3.7 Vulcanizing agents:

The process of cross-linking rubber molecules with sulphur is commonly referred to as vulcanization. To guarantee that certain medication delivery systems, such as implants, transdermal patches, or topical gels, keep their form and functionality after administration, they are employed to solidify or harden them. These agents are used to treat rubber materials. Examples of vulcanizing agents are materials like plasticizers, silicon rubber, chloroprene rubber, sulfur.

1.3.3.8 Residual monomer, polymer and oligomer:

An unreacted monomer during the process of monomers to polymers is known as residual monomers. Polymers have a large chain of monomers. An oligomer is a fragment that contains a few monomer elements. A molecule that can join with other

molecules to create an oligomer or polymer is called a monomer. A residual monomer is a little quantity of unreacted monomer molecules that are still present in a polymer following the completion of the polymerization process. Examples of agents like residual monomer, polymers and oligomers are polyethylene, polystyrene, proteins, biological polymers, carbohydrates, liquid paraffin.

1.3.3.9 Phthalates, nitrosamines and polyaromatic hydrocarbons (PAHs):

Phthalates are the esters of phthalic acids. A class of compounds known as phthalates is used to increase the flexibility and durability of plastics. The group of compounds having =NNO connected to the two organic groups are known as nitrosamine. Nitrates or nitrites react with specific amines to generate nitrosamines. A wide range of consumer goods, including processed meats, alcoholic beverages, cosmetics, and cigarette smoke, include nitrosamines and/or their precursors. The compound which has only hydrogen and carbon molecules connected to the aromatic rings. PAHs are made up of several fused aromatic rings, they have a special chemical structure that can be employed to create new drugs. Examples of phthalates, nitrosamine and polymeric hydrocarbon groups are as follows di-(2-ethylhexyl) phthalates, di-isononyl phthalate, benzyl butyl phthalate, di-isodecyl phthalate.

1.3.4 Classification of Extractables and Leachables

1.3.4.1 Organic compounds:

Generally, the compounds which have carbon are named as an organic compound. The safety and quality of the finished product may be impacted by this, which can occur through a variety of processes like oxidation, hydrolysis, or direct chemical reactions, depending on the kind of organic compound and the particular extractables and leachables chemicals involved. The organic molecule's reactivity with leachables can

be significantly influenced by the functional groups that are present on it. The interactions between organic compound and leachables can cause potential effects like loss of potency, changes in physical properties as it can change the formulation's viscosity, solubility, or appearance, forming harmful byproducts which are toxic or genotoxic chemicals produced by the API and leachables interactions. Examples of organic compounds are protein, carbohydrate, polystyrene, butane, pentane, acetaldehyde, amide, ether, benzene, ethane, naphthalene.

1.3.4.2 Inorganic compounds:

The compound which has deficiency of C-H bonds. Identification and measurement of inorganic extractables and leachables are frequently accomplished using methods such as inductively coupled plasma mass spectrometry (ICP-MS). Depending on the packaging material, metals such as silicon, iron, nickel, copper, chromium, aluminium, and nickel are examples of common inorganic extractables and leachables. Other examples of inorganic types of extractables and leachables are silica, ammonia, bromide, magnesium, sodium carbonate, carbon dioxide, barium oxide, borax, boric acid.

1.3.4.3 Volatile compounds:

This compound has high vapor pressure even at normal room temperature. A volatile solvent found in the medicine formulation may be able to remove volatile plasticizers from some plastic components found in drug containers. The evaluation of volatile extractables and leachables is especially crucial because of the nature of inhalation, which allows the patient to easily inhale volatile compounds from the packaging material. Rubber stopper volatile chemicals have the potential to seep into pharmaceutical products, particularly when exposed to harsh solvents or high

temperatures. Examples of volatile compounds are methanol, ethanol, chloroform, dioxane, alcohol, formaldehyde, methylene chloride, acetic acid, butanal, carbon disulfide.

1.3.4.4 Non-Volatile compounds:

Compounds which do not vaporize promptly are known as non- volatile compounds. Assessing non-volatile leachables is crucial for ensuring the safety of drug products, as they can potentially accumulate in the body over time if present in significant quantities. contaminants that are not readily volatile and can be detected using techniques like high-resolution liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Examples of non-volatile compounds are Butylated hydroxyl Toluene (BHT), Dimethyl Sulphoxide (DMSO).

1.3.4.5 Metal compounds:

The lattice of positive ions encircled by delocalized electron or the compound which has metallic bonds are basically a metal compound. There are several ways that metal ions can escape from a metal complex, including oxidation in that metal may develop an oxide layer upon exposure to oxygen, which may subsequently dissolve in the surrounding fluid. Complexation refers to some substances found in the environment that can combine with metal ions to produce stable complexes that aid in their dissolution. pH variations means the solubility of metal compounds can be strongly impacted by the solution's pH level. Examples- Aluminum, Zinc, Carbide, Copper Oxide, Iron Sulphide, Copper Chloride, Mercury Oxide, Cryolite.

1.4 Process for the Study of Extractables and Leachables

Here is the detailed process to conduct the study of extractables and leachables step wise. Starting from the very first classification about extractable and leachable. The first step is to decide that extractable and leachable belongs to which class. Followed by the extraction process selection as per the class of extractables and leachable, in that selection process selection of the solvents, extraction conditions and technique of the extraction plays an important role. According to the class of extractable and leachable the analytical technique should be selected. Various analytical techniques areavailable and from that selection of appropriate final or secondary analytical technique is important. Lastly the data are compared with the regulatory guidelines and check the standard as per data availability.

1.4.1 Process flow chart of extractables and leachables

Extractables and leachable are broadly classified into three types as per the above discussion. The one class is volatile or semi volatile materials, in that mainly organic compounds takes place, another class is of non-volatile compounds in that mainly organic compound takes place, but some inorganic compounds are also there, lastly the remaining class is of inorganic materials or the metal compounds.

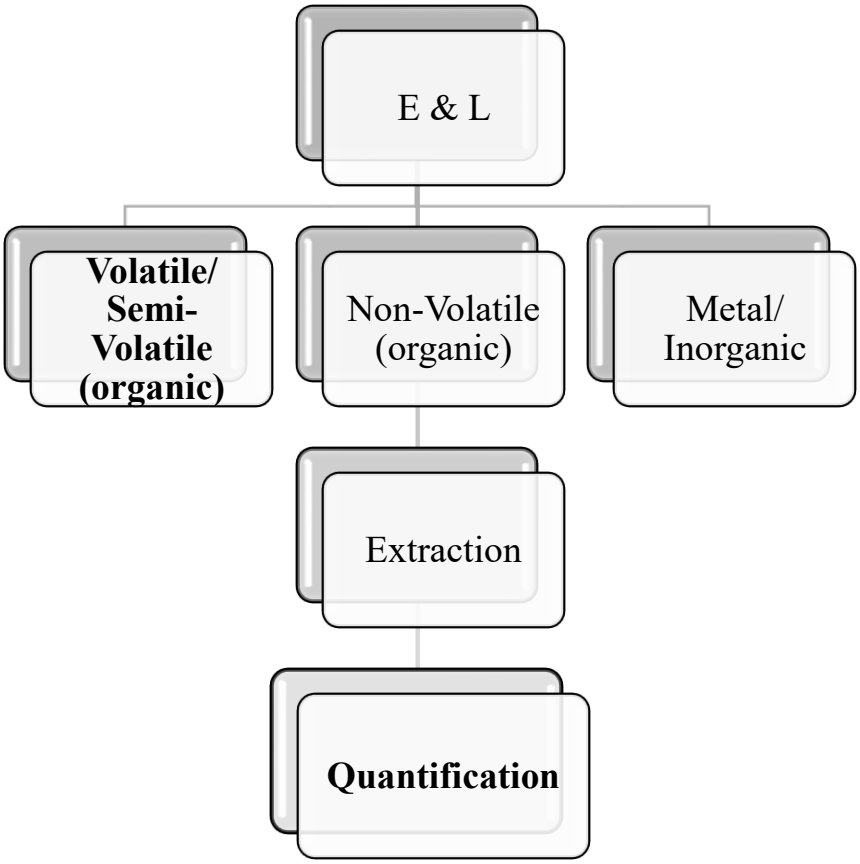


Fig 1.9: Process of Extractables and Leachables Study

1.4.2 Common Extraction Solvents, Techniques and Conditions for the Study of Extractables and Leachables

The extraction techniques like reflux, soxhlet, sealed vessel & solvent soaking, liquid-liquid extraction, sonication, solid phase extraction, sunlight, hot air oven, pH available for the study. With the use of numerous solvents and various extraction conditions available, few were selected for the present research work.

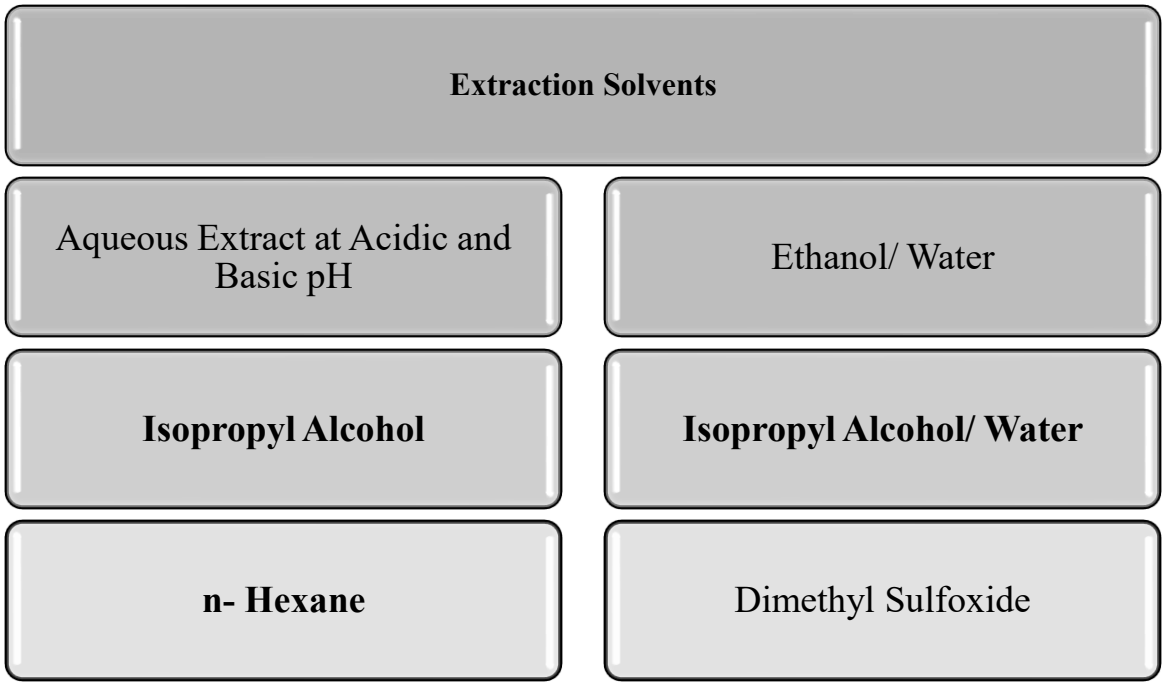


Fig 1.10: Common Solvents used for Extraction

Step: 3.2

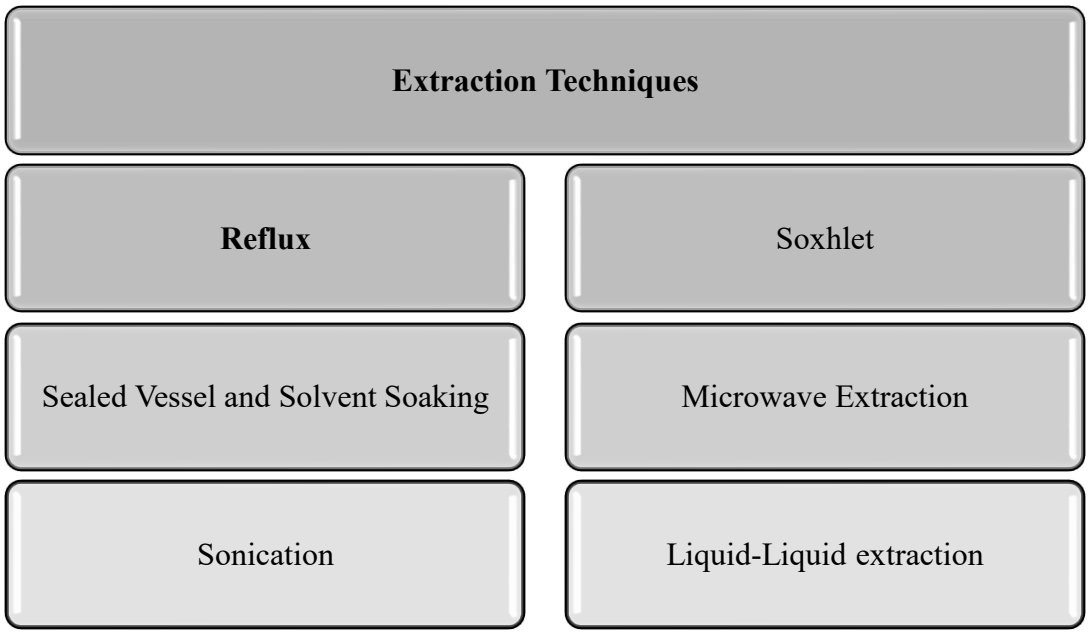


Fig 1.11: Common Techniques used for Extraction

Step: 3.3

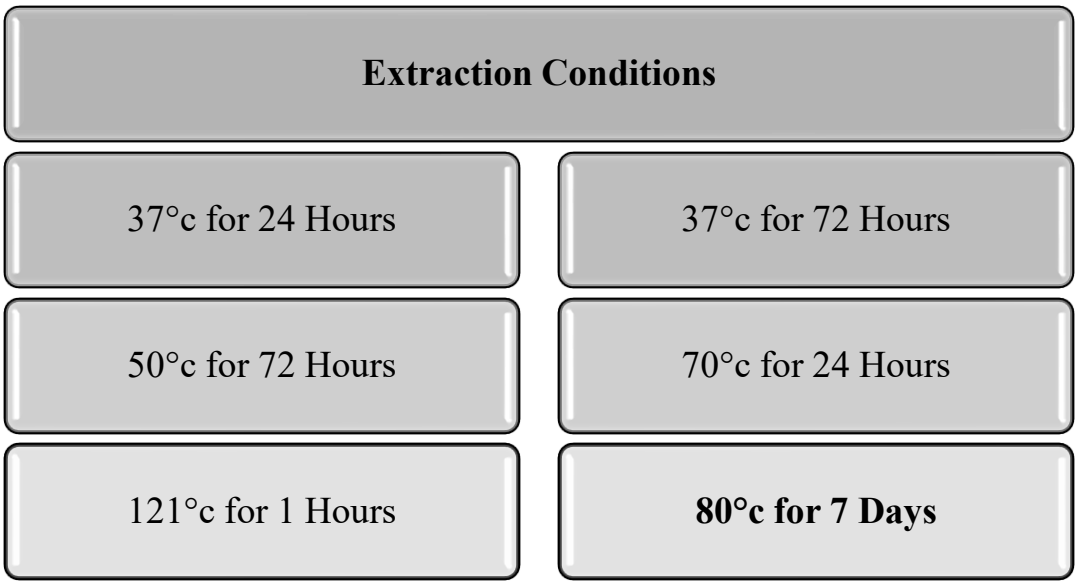


Fig 1.12: Common Extraction Conditions

1.4.3 Quantification of Extractables and Leachables

For the analysis of extractables and leachable various techniques are available. The basic techniques are termed as a primary technique of analysis. In those elementary techniques like IR, NMR, pH etc. techniques are performed. Afterwards the finest techniques come which are highly sensitive and accurate for the study. Techniques like LC, GC, ICP coupled with mass spectrometry can be performed for accurate results. Some examples are given below in that analytical technique mentioned which are suitable for the particular leachable class or material is provided.

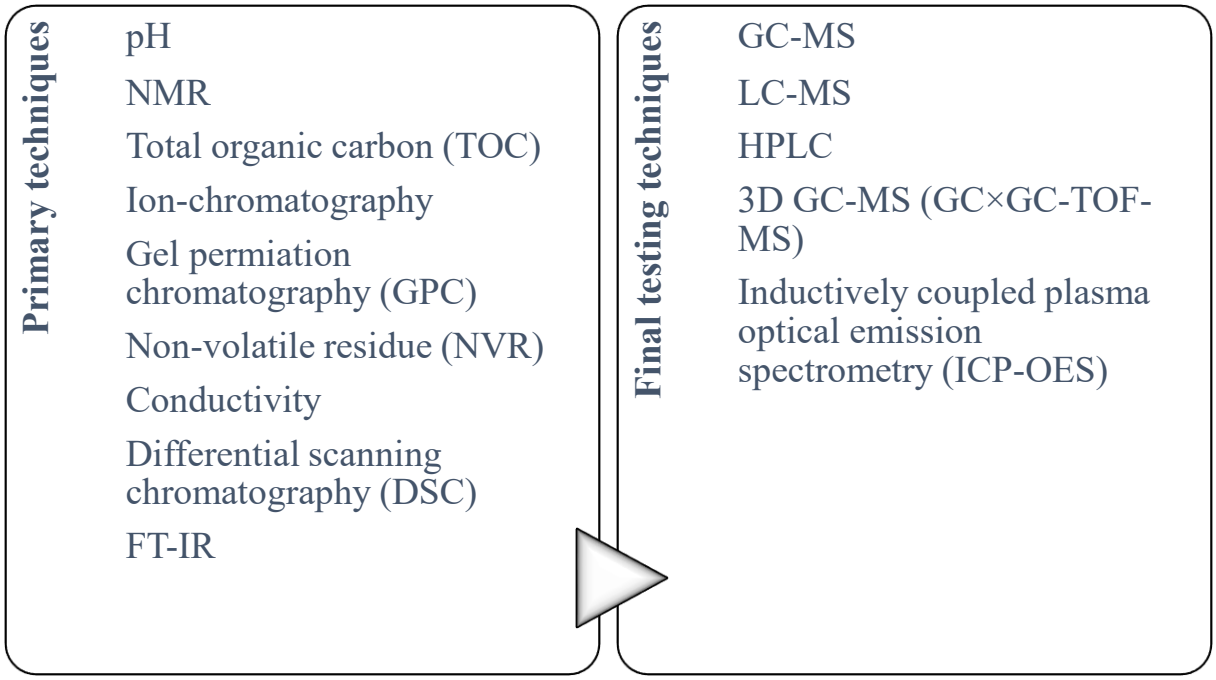


Fig 1.13: Classification of Analytical Techniques

Table 1.1: Quantification of extractables and leachables

Instruments	**Possible analyzed components**
Triple quadrupole MS (APCI) HPLC-UV	Fluorinated compound from polytetrafluoroethylene-coated seal
Single quadrupole MS Electrospray/APCI UPLC-DAD-UV	Non-volatiles & more polar species
Triple quadrupole MS ESCi UPLC-DAD-UV	Non-volatile &more polar species
GC-MS headspace autosampler	Volatile organic compound
GC-MS liquid injection	Semi-volatile organic species
GC-MS pyrolyzer	Polimeric &oligomeric compounds
LC-UV	Mercaptobenzothiozole
ICP-MS	Elements (particularly heavy metals)
GC-NCD	Nitrosamines from rubber components
GC-MS	Polycyclic aromatic hydrocarbon from rubber

1.5 Regulatory Studies:

The studies of regulatory compliance are carried out where compliance means meeting the requirements to a specification, rule, plan, standard and regulation. Regulatory compliance defines the objective that organizations desire to accomplish in their efforts to confirm that they are attentive of and take steps to fulfill with relevant regulations, policies and laws. Key components of regulatory requirements for extractables and leachables include conducting comprehensive testing to identify potential contaminants, evaluating their toxicity, and setting appropriate limits based on the drug product formulation and the route of administration. These requirements are primarily guided by FDA guidance documents, ICH Q3E guidelines, and pertinent USP chapters such as <1661>, <1663>, and <1664>. The main goal of these regulations is to ensure that packaging materials used for drugs and medical devices do not leach harmful substances into the product. To assess whether a detected leachable presents a safety risk, regulatory bodies frequently consult safety criteria such as the "qualifying threshold" (QT) and "safety concern threshold" (SCT). It is now evident that the implementation of regulatory guidance was necessary, and thorough testing of every component used in the creation of final products is now necessary. But testing for leachables and extractables can still be difficult and necessitates a substantial. The study of extractables and leachable helps to ensure that extractables and leachables comply with regulatory standards for container closure systems (CCS) as well as packing supplies in addition to facilitating a better knowledge of how to handle them during development. By detecting and reducing possible contaminants that might seep from drug packaging materials into the finished product, affecting its efficacy and safety, and by guaranteeing compliance with regulatory bodies such as the FDA and EMA during

drug approval processes, regulatory requirements for extractables and leachables are essential for.

1.5.1 PDA (Parenteral Drug Association) (PDA Guideline)

In order to ensure the highest level of patient safety when administering drugs via injection routes such as intravenous (IV), intramuscular (IM), and subcutaneous (SC), the Parenteral Drug Association (PDA) guidelines for parenteral drug administration offer thorough standards and best practices for the safe and sterile preparation, handling, and administration of injectable medications. These guidelines cover topics such as aseptic technique, facility design, personnel training, equipment selection, and quality control procedures. The Parenteral Drug Association (PDA) recommends that all container closure systems undergo extractables and leachables testing when it comes to parenteral drug administration. This is done to evaluate potential contaminants that may leach into the drug product from the packaging materials, with an emphasis on identifying and evaluating the safety of these compounds through a thorough risk assessment process. This includes conducting both extractables studies to identify potential contaminants and leachables studies to determine the amount of those contaminants that leach into the drug product under pertinent conditions, taking into account variables such as extraction solvents, temperature, and contact time. The PDA also advises adhering to established standards and recommendations from organizations such as USP and PQRI to ensure a robust evaluation of extractables and leachables in parenteral drug products. The Product Quality Research Institute (PQRI) is a non-profit consortium of organizations working together to generate and share timely, relevant, and impactful information that advances drug product quality and development. “When making Parenteral Drug Products, pharmaceutical companies are

faced with the need to further investigate the materials that will be in contact with the drug product, either during manufacturing, intermediate storage, storage in its final packaging, or during the delivery of the drug to the patient."

1.5.2 USFDA (USFDA Guideline)

According to the FDA's extractables and leachables (E&L) criteria, drug closures and containers must be safe and do not degrade the quality of the drug. Additionally, manufacturers must prove the safety of the materials used in production and packaging, according to FDA regulations. As per the guideline Closures and containers for drugs should not react, absorb, or combine with the substance. Final closures and containers should not cause the product to degrade or become less usable. Closures and containers must to be hygienic and devoid of any impurities that could leak out. Section 501(a)(3) of the US Food, Drug, and Cosmetic Act states that a drug is considered adulterated if any dangerous or harmful material, in whole or in part, is present in its container and could make the contents harmful to health. Biologics 21CFR600.11(h): All final closures and containers must be constructed of materials that will not accelerate the product's deterioration or make it less appropriate for its intended use. Surface solids, leachable pollutants, and other elements that could accelerate the product's deterioration or make it less appropriate for its intended purpose must be removed from all final containers and closures. Important factors for extractables and leachables (E&L) testing were covered in the US Food and Drug Administration (FDA) published in December 2023 advice on quality considerations for topical ophthalmic medication products. Also, in November 2024 guidelines about extractables and leachables data requirements before filling the ANDA were published by the FDA.

1.5.3 EMEA (EMEA Guideline)

In essence, the "EMEA Guidelines" are a set of guidelines designed to meet the specific needs of businesses operating in the "Europe, Middle East, and Africa" region as a whole. They provide a framework for consistent practices in marketing, operations, compliance, and other aspects of business activity within this diverse geographical area, considering the distinct legal and cultural landscapes of each sub-region. Finding those additives, such as plasticizers, catalysts, initiators, and antioxidants, is the goal of extraction studies. That might be extracted by the active substance in contact with the plastic material. Plastic materials utilized for nonsolid active ingredients and nonsolid dosage form container systems are thought to require extraction research. It is important to assess research on the interactions between active ingredients and plastic packaging materials. Migration studies are deemed “necessary when extraction studies have resulted in one or several extractables”.

1.5.4 Indian Pharmacopoeia (IP-2022)

A set of guidelines for medications produced and marketed in India is known as the Indian Pharmacopoeia (IP). On behalf of the Ministry of Health and Family Welfare, the Indian Pharmacopoeia Commission (IPC) publishes it. The India's official drug standards available in the Indian Pharmacopoeia (IP). There are 223 general chapters and 3,152 monographs in all. India's pharmaceutical quality is improved by IP. It also aids in the distribution, inspection, and licensing of pharmaceutical manufacture. To guarantee the quality and safety of medications, the IP establishes official criteria. The containers consist of polymers that do not include in their composition any substances that can be extracted by any contents in such quantities to alter the efficacy or stability of the product or to prevent a toxic hazard. Antioxidants, lubricants, plasticizers, and

impact modifiers are examples of additives; mold release agents and antistatic agents are not. The preparation ingredients that come into touch with the closure are not sufficiently adsorbed onto its surface to negatively impact the result. The closure should not give way to the product ingredients in amounts that could compromise its stability or provide a toxicity concern.

1.5.5 Schedule M (For product containers & closures) (D&C act)

Primarily passed in India in 1940, the Drugs and Cosmetics Act governs the import, production, distribution, and sale of pharmaceuticals and cosmetics. Its goal is to guarantee the products' safety, effectiveness, and quality by establishing standards for production and sale, as well as regulating the labelling and prescription requirements for different medications. In other words, the law controls the market to protect public health by preventing the sale of inferior or counterfeit pharmaceuticals and cosmetics. Indian pharmaceutical products must adhere to Good Manufacturing Practices (GMP), which are outlined in Schedule M of the Drugs and Cosmetics Act of 1945. Other than GMP schedule M covers regulations for pharmaceutical quality system (PQS), quality risk management (QRM), product quality review (PQR), qualification and validation of equipment, computerized storage system for all drug products, manufacturing process quality control, manufacturing-facility requirements and maintenance, personnel training. It addresses the specifications for manufacturing facilities, machinery, and real estate. All containers and closures planned for practice shall obey the pharmacopeial requirements. Strict adherence to appropriate, validated test procedures, sample sizers, specifications, cleaning and sterilization procedures, if applicable, is required to guarantee that the drug's quality or purity is not adversely affected by reactive, additive, absorptive, or leaching substances.

1.5.6 ICH Q3E (ICH Guideline)

International standards for the creation and approval of safe and efficient medications are known as ICH recommendations. These recommendations were developed by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). A guideline called ICH Q3E is being created to evaluate and manage leachables and extractables (E&L). The goal of the guideline is to encourage global harmonisation. Important milestones like conducting stability studies, establishing pertinent limits for impurity testing, and adopting a more flexible approach to pharmaceutical quality based on Good Manufacturing Practice (GMP) risk management are examples of harmonisation accomplishments in the quality sector. A recently added guideline under the export working group about extractables and leachables is to produce the Q3E Guideline on the assessment and control of extractables and leachables (E&L), the Q3E EWG was formed. It is anticipated that this will help both applicants and regulators by emphasizing important details and enhancing the clarity of requirements for pharmaceuticals, including parts for drug delivery devices.

1.6 Introduction to GC-MS/MS: (Shimadzu Corporation, Japan, TQ8040)

Leachables and extractables are studied using a variety of methods, including LC-MS/MS, GC-MS/MS, ICP-MS, and others. For volatile organic components, GC-MS/MS is the most sensitive of these methods. In this work, extractables and leachables are studied using GC-MS/MS. By analyzing complex chemical mixtures and identifying their individual components based on their unique mass-to-charge ratio, Gas Chromatography-Mass Spectrometry (GC-MS) is a powerful analytical technique that combines the separation capabilities of gas chromatography (GC) with the

identification power of mass spectrometry (MS). This technique enables the precise identification and quantification of volatile compounds within a sample and is frequently used in fields such as environmental monitoring. GC-MS can be used to study liquid, gaseous or solid samples. Analysis begins with the gas chromatograph, where the sample is effectively vaporized into the gas phase and separated into its various components using a capillary column coated with a stationary (liquid or solid) phase. The compounds are propelled by an inert carrier gas such as helium, hydrogen or nitrogen. As components of the mixture are separated, each compound elutes from the column at a different time based on its boiling point and polarity. The time of elution is referred to as a compound's retention time. GC has the capacity to resolve complex mixtures or sample extracts containing hundreds of compounds.

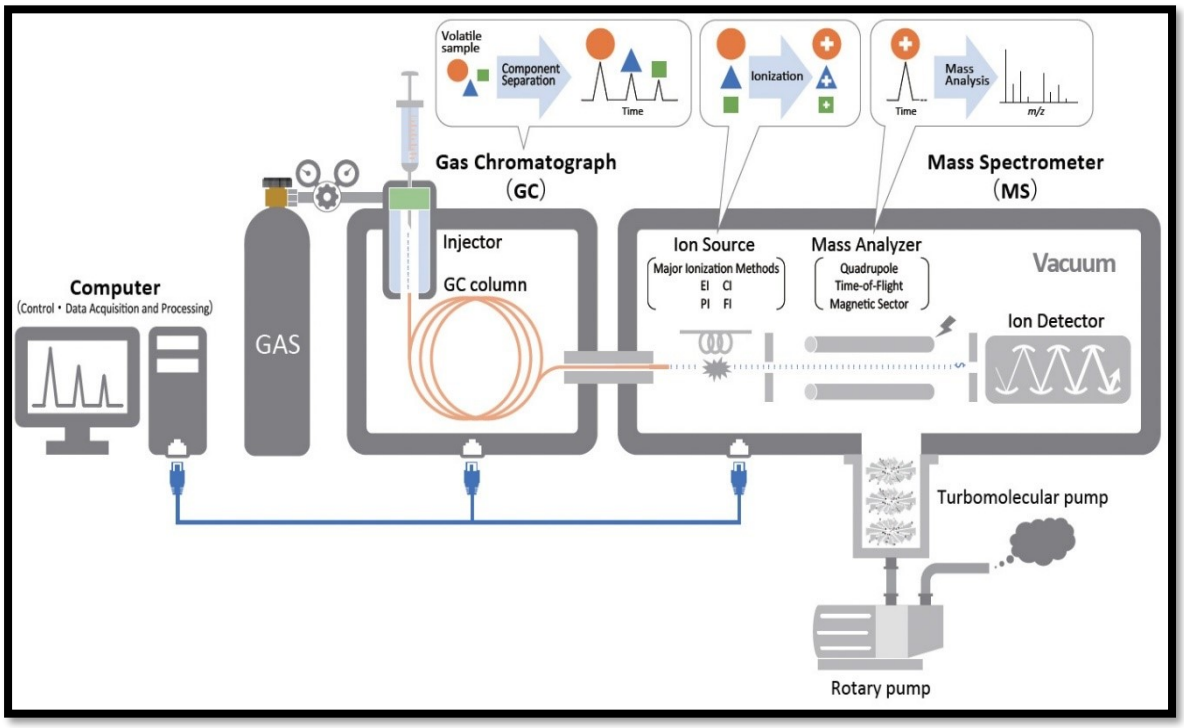


Fig 1.14: Block Diagram of GC-MS Instrument

1.6.1 Instrumentation of GC-MS/MS:

The analytical method known as gas chromatography-mass spectrometry is hyphenated. As the name suggests, it essentially consists of combining two methods to create a single approach to chemical mixture analysis.

Mass spectrometry characterizes each constituent separately, while gas chromatography separates the components of a mixture. A solution comprising multiple potential compounds can be evaluated qualitatively and quantitatively by combining the two methods.

When a sample is put into a GC column, a carrier gas (such as helium) carries the components through, separating them according to their boiling points. An inert vapor, such as argon, nitrogen, or helium, serves as the moving segment in gas chromatography. A chemical that particularly draws components to a sample mixture is often the stationary phase. Typically, it is housed in a column, which is a tube of some kind. Glass or stainless steel are the materials used to make columns, and they come in a variety of sizes. A distinct "fingerprint" for identification is created when the separate components reach the mass spectrometer, where they are ionised, broken up, and examined according to their mass-to-charge ratio. The rate of interaction varies for each of the mixture's compounds. Less interactive elutes will leave the column last, while those that interact the fastest will do so first. Altering the column's temperature or the vehicle's pressure may be necessary for the separation process. To guarantee that only volatile chemicals are examined, the sample is usually prepared via headspace sampling or liquid extraction. The sample is injected into the GC column where it vaporizes and is carried by carrier gas. Typically, a micro needle is used to inoculate the trial through the elastic septum into the flash vaporizer port at the column head.

Direct mode or split less can be used for injection splitting. Different compounds in the mixture travel through the column at different speeds based on their interaction with the stationary phase lining the column, effectively separating them. The individual chemicals enter the electron impact detector after eluting from the column. They disintegrate into pieces after being subjected to a stream of electrons. Both big and little parts of the original molecules may be included in these fragments. After being separated, the chemicals are put into the mass spectrometer and ionized, usually by electron impact (EI), which fractures the molecules. The mass-to-charge ratio of the ionized fragments is used to separate them, producing a mass spectrum that, when compared to a reference library, can be used to identify the molecule. When MS is thrown at GC, a potent analytical tool is produced. It is possible to inject an organic solution into the device, which will detect and separate the various components. Furthermore, following meticulous calibration, the amounts of each component can be ascertained. In present work GC-MS/MS technique was used because it is a highly sensitive instrument, or an analytical technique as compared to other analytical techniques. Other than this it has an ability to analyze a volatile compound which is most required. Also, it has the advantage of a library which can identify the peak and provide compounds name so it may be helpful for identification. The major application of the instrument is finding and quantifying organic trace elements in a large sample. The measurement of chemical concentrations in fuels, consumer goods, medications, food, and the environment. Recognizing organic substances like sterols, alkanes, fatty acids, and alkenones.

- **Advantages:**

- It is an efficient technique.
- Highly sensitive

- Highly accurate quantitative analysis
- Requires small amount of samples
- Reliable and relatively simple.

➢ **Disadvantages:**

- It is solely applicable to volatile samples.
- It is not suitable for thermos labile samples.
- It requires elaborate instruments such as mass spectrometry for confirmation of peak identification.

1.7 Introduction to Cell line (Jenke, D., et, al.)

Studies on cytotoxicity are crucial because they provide insight into how chemicals impact the viability and health of cells. Research on cancer, toxicity, and medication development all make use of this data. Cytotoxicity studies aid in the development of novel medications by ensuring their efficacy and safety. In safety of medical equipment these cytotoxicity tests aid in ensuring the usability of medical gadgets. Also, the research work on cancer tis study give researchers insight into how drugs impact the viability and health of cells and on toxicology studies it gives researchers insight into how drugs impact the health and survival of cells. Toxicological study can be performed through two techniques. *In vivo* and *in vitro* studies are the part of toxicological studies. In the present work *in vitro* study was adopted. The concept of cell line study was newly discovered to perform the toxicity testing. Cell line study was performed by using two different cell lines for the toxicity testing. Cell culture is an indispensable technique and one of the major tools to understand the structure and function of the cells. It is being employed to obtain mechanistic data pertaining to life science investigations such as cancer, infertility, toxicological studies and metabolic disorders. Cell Line: Produced

from initial cultures that have a finite lifespan, during passage, the cells with the highest potential for expansion are predominant, leading to a certain level of genotypic and phenotypic homogeneity in the population. Toxicity Testing: The effects of new medications, cosmetics, and chemicals on the growth and survival of various cell types, particularly liver and kidney cells, are investigated using animal cell culture. The maximum allowable dosage of a novel medication is also ascertained using cultured animal cells. In conclusion, cytotoxicity tests play a critical role in the betterment of medications. They aid in our comprehension of the possible effects that therapies may have on our cells, which is essential for guaranteeing the efficacy and safety of novel medications.

1.8 Introduction to Selected Extractables and Leachables Profiles

For the present work a total of eight analytes have been selected as extractables and leachables namely naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, alpha methyl styrene, diethyl azelate and tributyrin. Amongst the first five are naphthalene, acenaphthene, anthracene, fluoranthene and pyrene have belonged to the rubber materials category which are mainly used in the formulation of rubber and closure type of materials for the pharmaceutical products. The other three are used as an additive mostly and in the preparation of inks or labeling materials as well. According to the categories analytes were divided into two groups for the experiment. Group one is of five analytes separated by one method and another method developed for group two which is for three analytes. On the basis of review of literature, it was found that there is no single method available for the separation of selected analytes. After development both the methods were applied successfully to the selected liquid pharmaceutical formulations available in the local market. Lastly, the cytotoxicity study of all eight

analytes have been performed using two different cell lines. Here is the detailed information including physicochemical profile about all the selected extractables and leachables like structure, IUPAC name, molecular weight, formula, properties etc. have been mentioned in the below mentioned tables.

Table 1.2: Physicochemical Profile of Naphthalene

(https://pubchem.ncbi.nlm.nih.gov/compound/Naphthalene**)**

Parameters	**E&L Profile**
Structure	
IUPAC Name	Naphthalene
Empirical Formula	$C_{10}H_8$
Molecular Weight	128.17 g/mol
Properties	Naphthalene appears as a white crystalline volatile solid with a strong coal-tar odor.
E&L Category	Used as a moth repellent, fumigant, lubricants, and to make other chemicals, and for many other uses.
Melting Point	80.2 °C
Boiling Point	218 °C
Solubility	insoluble in water, very soluble in ether and other organic solvents.
Storage Condition	Store in an area without drain or sewer access. Provision to contain effluent from fire extinguishing.
Toxicity Effect	Nervous system and Respiratory system

Table 1.3: Physicochemical Profile of Acenaphthene

(https://pubchem.ncbi.nlm.nih.gov/compound/Acenaphthene**)**

Parameters	**E&L Profile**
Structure	
IUPAC Name	1,2-dihydroacenaphthylene
Empirical Formula	$C_{12}H_{10}$
Molecular Weight	154.21 g/mol
Properties	Acenaphthene appears as white needles.
E&L Category	Used to make dyes, pharmaceuticals, insecticides, fungicides, and plastics.
Melting Point	93 °C
Boiling Point	279 °C
Solubility	Soluble in hot alcohol and insoluble in water.
Storage Condition	Store in an area without drain or sewer access.
Toxicity Effect	Hepatic system

Table 1.4: Physicochemical Profile of Anthracene

(https://pubchem.ncbi.nlm.nih.gov/compound/Anthracene**)**

Parameters	**E&L Profile**
Structure	
IUPAC Name	Anthracene
Empirical Formula	$C_{14}H_{10}$
Molecular Weight	178.23 g/mol
Properties	Anthracene is a white to yellow solid with a weak aromatic odor.
E&L Category	Used to make synthetic fibers and plastics and in semiconductor research.
Melting Point	218 °C
Boiling Point	342 °C
Solubility	Insoluble in water,
Storage Condition	Keep the container tightly closed in a dry and well-ventilated place. Protect from exposure to light.
Toxicity Effect	Skin, respiratory tract, bladder, stomach, and kidney.

Table 1.5: Physicochemical Profile of Fluoranthene

(https://pubchem.ncbi.nlm.nih.gov/compound/Fluoranthene**)**

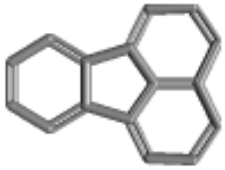
Parameters	**E&L Profile**
Structure	
IUPAC Name	Fluoranthene
Empirical Formula	$C_{16}H_{10}$
Molecular Weight	202.25 g/mol
Properties	Fluoranthene appears as light-yellow fine crystals.
E&L Category	Used to make drugs and fluorescent dyes; Also used as a stabilizer in epoxy resins and in electrically insulating oils.
Melting Point	110.2 °C
Boiling Point	384 °C
Solubility	Soluble in ethanol, ether, benzene, chloroform and carbon disulfide, insoluble in water.
Storage Condition	Protect this material from exposure to light and store it in a refrigerator.
Toxicity Effect	Hepatic system and Urinary system.

Table 1.6: Physicochemical Profile of Pyrene

(https://pubchem.ncbi.nlm.nih.gov/compound/Pyrene)

Parameters	E&L Profile
Structure	
IUPAC Name	Pyrene
Empirical Formula	$C_{16}H_{10}$
Molecular Weight	202.25 g/mol
Properties	Pyrene is a colorless solid, solid and solutions have a slight blue fluorescence.
E&L Category	It is a by-product of the pyrolysis of organic matter and is present in coal tar distillates, diesel exhaust, automobile exhaust, tobacco smoke, barbecue smoke, wood smoke, lake sediments, waste oils, and sewage.
Melting Point	151 °C
Boiling Point	404 °C
Solubility	Soluble in ethanol, ethyl ether, benzene, toluene; slightly soluble in carbon tetrachloride, insoluble in water.
Storage Condition	Store in a flammable materials storage area. Store in a cool, dry place.
Toxicity Effect	Urinary system

Table 1.7: Physicochemical Profile of Alpha Methyl Styrene

(https://pubchem.ncbi.nlm.nih.gov/compound/alpha-METHYL-STYRENE)

Parameters	**E&L Profile**
Structure	
IUPAC Name	Prop-1-en-2-ylbenzene
Empirical Formula	C_9H_{10}
Molecular Weight	118.18 g/mol
Properties	Isopropenylbenzene appears as a colorless liquid with a characteristic odor.
E&L Category	Used as a solvent and to make other chemicals.
Melting Point	-23 °C
Boiling Point	164 °C
Solubility	Insoluble in water, soluble in benzene, chloroform, acetone and carbon tetrachloride.
Storage Condition	Store in an area without drain or sewer access. Fireproof. Well closed. Separated from strong oxidants.
Toxicity Effect	Inhalation, ingestion, skin and/or eye contact

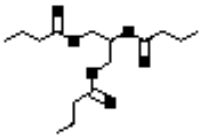
Table 1.8: Physicochemical Profile of Diethyl Azelate

(https://pubchem.ncbi.nlm.nih.gov/compound/Diethyl-azelate)

Parameters	E&L Profile
Structure	
IUPAC Name	Diethyl nonanedioate
Empirical Formula	$C_{13}H_{24}O_4$
Molecular Weight	244.33 g/mol
Properties	Colorless liquid
E&L Category	Flavoring agents and food additives
Melting Point	15-16 °C
Boiling Point	172 °C
Solubility	Soluble in acetone, benzene, chloroform, acetone and carbon tetrachloride and other organic solvents.
Storage Condition	Store in an area without drain or sewer access.
Toxicity Effect	Potential allergic reactions, especially in sensitive individuals, could manifest as skin rash, itching, or in severe cases, anaphylaxis.

Table 1.9: Physicochemical Profile of Tributyrin

(https://pubchem.ncbi.nlm.nih.gov/compound/Tributyrin)

Parameters	E&L Profile
Structure	
IUPAC Name	2,3-di(butanoyloxy)propyl butanoate
Empirical Formula	$C_{15}H_{26}O_6$
Molecular Weight	302.36 g/mol
Properties	Colorless oily liquid with fruity, buttery odor and bitter taste.
E&L Category	Used in synthetic flavoring substance and adjuvant.
Melting Point	- 75 °C
Boiling Point	307 °C
Solubility	soluble in acetone, benzene, very soluble in alcohol and ether, insoluble in water.
Storage Condition	Store in an area without drain or sewer access.
Toxicity Effect	Diarrhea, headache, abdominal cramping, nausea, anemia, constipation, azotemia, lightheadedness, fatigue, rash, alopecia, odor, dysphoria, and clumsiness.

Chapter- 2

Aim and Objectives

Aim:

- The aim is to identify and quantify the undesired substances in pharmaceutical liquid formulations (introduced through packaging/ container closure system) using chromatographic techniques and to conduct their safety assessment.

Objectives:

- To identify the most prevailing extractables and leachables.
- To develop and validate gas chromatography- mass spectroscopic methods for the identification of extractables and leachables.
- To perform the invitro toxicity study for the selected extractables and leachables.

Chapter- 3

Review of Literature

Table: 3.1 Review of Literature

Sr. No	Name of journal and Title	Instruments	Findings	Reference
1.	PDA J Pharm Sci Technol. Oct. 2013 Extractables Characterization for Five Materials of Construction Representative of Packaging Systems Used for Parenteral and Ophthalmic Drug Products	HPLC/ UV/ MS, GC/ FID, GC/ MS, ICP/ MS	Five materials: PVC, LDPE, PC, COC, brominated-isobutylene-isoprene rubber	Jenke, D., et, al.,
2.	Eur. J. Pharm. Biopharm. Dec. 2016 Matrix effect on leaching of Bisphenol A diglycidyl ether (BADGE) from epoxy resin based inner lacquer of aluminium tubes into semi-solid dosage forms	RP-HPLC	The contamination of the medicinal product by BPA, BADGE and BADGE derivatives can be precluded by using aluminium tubes with an internal lacquer with a low degree of unbound polymerisation residues	Lipke, U., et, al.,
3.	J. Environ. Manag Jan.2015 Bisphenol-A removal in various wastewater treatment processes: Operational conditions, mass balance, and optimization	LC/MS/ MS, GC	Removal of BPA is more effective in summer as compare to winter because of temperature difference	Guerra, P., et, al.,
4.	Chem Eng J Nov.2017 Transformation of bisphenol A during chloramination in a pilot-scale water distribution system: Effect of pH, flow velocity and type of pipes	HPLC, EI/GC/MS GC/ ECD	Complete degradation of BPA after chloramination required >9 h. At Cl/N mass ratios of 3, 4 or 6, the degradation rate was appreciably greater in the WDS than in the DW	He, G., et, al.,
5.	J. of water res. Nov. 2007 Sensory aspects and water quality impacts of chlorinated and chloraminated drinking water in contact with HDPE and cPVC pipe	GC/ MS	The research demonstrates the impact that different types of premise plumbing materials can have on water quality aesthetics	Heim, T.H., et, al.,

6.	Eur. Polym. J. Apr. 2005 Nonisothermal crystallisation, melting behavior and wide-angle X-ray scattering investigations on linear low-density polyethylene (LLDPE)/ethylene vinyl acetate (EVA) blends: effects of compatibilisation and dynamic crosslinking	DSC, Xray	Dicumyl peroxide (DCP-crosslinking) is more effective in EVA than in LLDPE.	Moly, K.A., et, al.,
7.	J. of anal. chem. June 2016 Detection & identification of leachables in vaccine from plastic packaging materials using UPLC-QTOF MS with self-built polymer additives library	UPLCQTOF/MS	The concentrations of leachables in vaccine and the intermediates ranged from 0.85 to 21.91 µg/L	Zhang, Y., et, al.,
8.	PDA J Pharm Sci Technol Oct. 2015 Creating a holistic extractables & leachables program for biotechnology products	GC/FID, GC/MS, LC/MS, ICP/MS, NMR	Biotech. Pdts.: siloxane, fatty acid, amides, methacrylates, PE, PP, PC, FEP, ETFE	Li, K., et, al.,
9.	PDA J Pharm Sci Technol Nov. 2013 The Product Quality Research Institute (PQRI) Leachables and Extractables Working Group Initiatives for Parenteral and Ophthalmic Drug Product (PODP)	-	Guideline (review)	Paskiet, D., et, al.,
10.	PDA J Pharm Sci Technol Nov. 2012 Interactions between Therapeutic Proteins and Acrylic Acid leachable	HPLC/MS/MS, RPLC	IgG2 antibody covelently modified by acrylic acid (0.02-0.3%)	Liu, D., et, al.,
11.	PDA J Pharm Sci Technol Nov. 2014 A compilation of safety impact information for extractables associated with materials used in pharmaceutical packaging, delivery, administration & manufacturing system	-	Review	Jenke, D., et, al.,
12.	J Analyst, 1977 Communication Determination of cyclohexanone in intravenous	GC-MS	Determination of Cyclohexanone from PVC containing IV bags.	Ulsaker, G.A. and Korsnes, R.M.,

	solutions stored in PVC bags by gas chromatography			
13.	J. of Pharm and Biomed Anal Non-volatile extractable analysis of prefilled syringes for parenteral administration of drug products	UHPLC-MS	Column – ORTECS UPLC C18 column (1.6 m; 2.1 mm × 50 mm) Mobile phase – (gradient flow) ammonium formate (pH 3.0; 50 mM) and acetonitrile	Dorival-García, N., et. al.,
14.	J of Pharma and Biomed Anal Strategy for identification of leachables in packaged pharmaceutical liquid formulations	GC-MS	Column - HP-5MS, 30m×0.25mm i.d.×0.1 m film thickness Carrier gas – helium Flow rate - 1ml/min oven column temperature - initiated at 40◦C, held for 1min, raised to 200◦C at 10◦C/min, and held at that temperature for 5min. Mass transition (m/z) - 197	Pan, C., et. al.,
15.	Pharm Res Best Practices for Extractables and Leachables in Orally Inhaled and Nasal Drug Products: An Overview of the PQRI Recommendations	-	Extractable and leachable study for oral inhaled and nasal drug product.	Norwood, L., et. al.,
16.	Mass Spectrometry Reviews The role of mass spectrometry and related techniques in the analysis of extractable and leachable chemicals	-	Analytical techniques HPLC GC-MS Mass Spectroscopy IR spectroscopy	Sica, P., et. al.,
17.	J of Anal Chem Structure Elucidation of an Artifact Discharging from Rubber-Based Vial Closures by Means of Gas Chromatography/Tandem Mass Spectrometry	GC-MS/MS	column –CP-Sil 8ms capillary column (30 m, 0.25-mm i.d., film thickness 0.25 ím Carrier gas – Helium Flow rate - 1 mL min-1 Oven temperature – started at 60 °C (held for 3 min), was then raised at 3 °C/min to 110 °C (held for 0.33 min), and was finally raised at 10 °C/min to	Kapp, T., Vetter, W.

			270 °C (held for 24 min).	
18.	J of Pkg Tech and Res Systematic Approaches on Extractable and Leachable Study Designs in Pharmaceuticals and Medical Devices: A Review	-	Regulatory guidance Extractable study Leachable study Extraction method Analytical techniques Toxicology assessment	Sharma, N., et. al.,
19.	Int J of Ph'ceutics Leachables from plastic materials in contact with drugs. State of the art and review of current analytical approaches.	-	Impurity Extractable and leachables Extractables and leachables guidelines	Cuadros-Rodriguez, L., et. al.,
20.	J of Pharma and Biomed Anal Solid phase extraction in tandem with GC/MS for the determination of semi-volatile organic substances extracted from pharmaceutical packaging/delivery systems via aqueous solvent systems	GC-MS	Column –35% Diphenyl/65% dimethyl polysiloxane Column dimensions 30 m × 0.25 mm, 0.25 m film thickness Carrier gas – Helium Flow rate - 1.0 mL/min Oven temperature – initial 40◦C for 5 min rise 10◦C/min Final temperature 300◦C for 4 min	Zdravkovic, A.
21.	J of Pharma and Biomed Anal Structural identification of extractables from rubber closures used for pre-filled semisolid drug applicator by chromatography, mass spectrometry, and organic synthesis	HPLC	Column – Prodigy ODS (3), 3 m, 15cm×0.32cm Mobile phase – (gradient) acetonitrile Flow rate - 0.6ml/min Wavelength - 220nm	Zhang, F., et. al.,
22.	ACS Omega Toxicological and Sedative Effects of Chipilin (Crotalaria longirostrata) Leaf Extracts Obtained by Maceration and Supercritical Fluid Extraction	GC-MS	Column –HP5-MS (30m×0.25 µm i.d., ×0.25mmfilm thickness Carrier gas – Helium Flow rate - 1.0 mL/min Oven temperature – initial 50°C for 1 min, followed by 30°C/min until 280°C for 10min, and then at 15°C/min until reaching 300°C for 4min.	Hernández-Reyes, A., et. al.,
23.	J of Pharma and Biomed Anal Stir bar sorptive extraction combined with GC–MS/MS for	GC-MS/MS	Column –DB-5ms-ultra inert capillary column of 40 m length, 0.18	Armstrong, L., et. al.,

	determination of low level leachable components from implantable medical devices		mm internal diameter and 0.18 m film thickness. Carrier gas – Helium Flow rate - 1 ml/min Oven temperature – 70◦C (1 min hold) then 10◦C/min to 220◦C then 25◦C/min to 275◦C (2 min hold).	
24.	Reviews in Sep Sci Evaluation of Extraction Conditions for Volatile Extractables from Polypropylene, PBT Resins, and Chlorobutyl Rubber Elastomers Using a Variety of Solvents and Extraction Techniques	GC-MS	Column –Restek Rxi 5 MS column (40 m x 0.18 mm with 0.18 µm film thickness Carrier gas – Helium Flow rate - 1.1 mL/min Oven temperature – 50°C (1-min ute hold) to 325°C at 10°C /min (3-minute hold). Mass transition (m/z) - 50-550 m/z range	Norwood, D., et. al.,
25.	J of Anal Let Determination of Acrylic Resin Monomers in Food Packaging Paper by Gas Chromatography–Tandem Mass Spectrometry (GC-MS/MS) with Formic Acid as a Protective Agent	GC-MS/MS	Column – 6%cyanopropylbenzene-94%dimethylsiloxaneultra-inert column (30m 0.32mm i.d. coated with 1.8lm film thickness Carrier gas – Helium Flow rate - 2mLmin 1 Oven temperature – initial 30◦C for 5 min rise 10◦C/min Final temperature 200◦C for 10 min	Wang, X., et. al.,
26.	J of Anal and Bio Anal Chem Simultaneous determination of the free and total forms of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate in human urine by gas chromatography-mass spectrometry	GC-MS	Column –DB-5MS capillary column (30.0 m length × 0.250 mm i.d., 0.250 µm film thickness Carrier gas – Helium Flow rate - 0.8 mL min−1 Oven temperature – initial 80^{0} C rise 180^{0} C	Shin, C., et. al.,

			at 10 °C/min than 240 °C at 3 °C/min Mass transition (m/z) - 135	
27.	J of Composites Sci Quantification of Irgafos P-168 and Degradative Profile in Samples of a Polypropylene/Polyethylene Composite Using Microwave, Ultrasound and Soxhlet Extraction Techniques	GC-MS	column –DB-5 ms of 30 m length, 0.25 mm internal diameter, and 0.25 µm film Carrier gas – Helium Flow rate - 1 mL min-1 Oven temperature – started at 60 ◦C for 3 min and then increased to 300 ◦C at a rate of 10 ◦C per minute, maintaining this temperature for 15 min.	Hernández-Fernández, J., et. al.,
28.	Patent no.: EP3215246B1 Title: A method for the removal of leachables and/or extractables	-	It has been found that the amount of leachables/extractables in the target molecule preparation re- sulting from the use of disposable equipment can be re moved or reduced by filtration through activated carbon devices.	Skudas, R., et. al.,
29.	Patent no.: US 10793592 B2 Title: Activated carbon for the removal of leachables and / or extractables	-	The present invention relates to the purification of target like recombinant and/or biotherapeutic proteins. Activated carbon can be used to remove leachables and / or extractables resulting from disposable equipment employed in the process	Skudas, R., et. al.,
30.	Patent no.: US 10088457 B2 Title: Low pollutant dialysis solution	-	The present invention relates to a low pollutant dialysis solution and a method for analyzing a dialysis solution. The present invention further relates to methods of validating dialysis	Athenstaed, B.

			solution batches, primary packaging material and the optimization of sterilization procedures	
31.	Patent no.: CN108463493B Title: Polymeric substrate having a surface with reduced biomolecule adhesion and thermoplastic articles of such substrate	-	Polymeric substrate having a surface with reduced biomolecule adhesion and thermoplastic articles of such substrate. Glass and plastic cand produce extractable and leachable.	A·塔哈, J·T·费尔特斯
32.	Patent no.: JP5990832B2 Title: Dexmedetomidine premix formulation	-	Uncoated infusion stoppers were evaluated. During the feasibility test, potency and a reduction in the extractables of the stopper were observed	シダーグレン プロロエシエ

Chapter- 4

Materials and Methods

4.1 Various lists including analytes, materials, apparatus, equipment and parts of instrument

Table: 4.1 List of standard extractables and leachables

Sr. no.	Materials	Manufacturer	Grade
1	Acenaphthene	Sigma Aldrich	Analytical Standard
2	Anthracene	Sigma Aldrich	Analytical Standard
3	Naphthalene	Sigma Aldrich	Analytical Standard
4	Fluoranthene	Sigma Aldrich	Analytical Standard
5	Pyrene	Sigma Aldrich	Analytical Standard
6	Alpha- Methyl Styrene	Sigma Aldrich	Analytical Standard
7	Diethyl Azelate	Sigma Aldrich	Analytical Standard
8	Tributyrin	Sigma Aldrich	Analytical Standard

Table: 4.2 List of chemicals, reagents, and solvents

Sr. no.	Materials	Grade	Manufacturer
1	Acetone	HPLC	Loba chemie, India
2	Methanol	HPLC	Rankem, India
3	Water	Milli Q	Millipore, USA
4	Dichloromethane	AR	Rankem, India
5	Isopropyl alcohol	AR	Rankem, India
6	n-Hexane	AR	Rankem, India
7	Potassium hydrogen phosphate	AR	Rankem, India

8	Hydrochloric acid	AR	Rankem, India
9	Ammonium chloride	AR	Rankem, India
10	Ammonia	AR	Rankem, India
11	Dimethyl sulfoxide	AR	Rankem, India

Table: 4.3 List of glassware and apparatus

Sr. no.	Glassware / Apparatus	Grade / Class (Volume in mL)	Manufacturer/ Supplier
1	Volumetric flask	Appropriate volumes (100, 50, 25, 10)	Borosil Glassworks Ltd.
2	Glass beakers	Appropriate volumes	Borosil Glassworks
3	Measuring cylinder	Appropriate volumes	Borosil Glassworks
4	Glass vials	--	Tarsons
5	Glass vial caps	--	Tarsons

Table: 4.4 List of equipment and accessories

Sr. no.	Equipment / Accessories	Capacity	Model	Manufacturer/ Company
1	Digital analytical balance	10mg to 220g	AUX220	Shimadzu corporation, Japan
2	Micropipette	10-100µL, 20-200µL, 100-	--	Eppendorf, Germany
3	Micropipette tip	As per appropriate volume (100µL,	--	GENAXY filter barrier tips, India
4	Vortex mixer	100 RPM	RM02 plus	REMI corporation
5	Water purification		Milli-Q	Merck Millipore, USA

6	Digital pH meter	-	Cyberscan pH tutor (pH/˚c meter)	EUTECH instruments pvt.
7	FTIR	-	Cary 630	Agilent, USA

Table: 4.5 List of Gas Chromatographic- Mass Spectroscopic Instrumentation Specifications

Sr. no.	Instrument / Apparatus	Brand/Model	Manufacturer/ Supplier
1	MS detector	GCMS-TQ8040	Shimadzu Corporation, Japan
2	Auto sampler	AOC-20 S Shimadzu	
3	Auto Injector	AOC-20i	
4	Column oven	CTO-20AC	
5	Degasser	DGU – 20A3	
6	Library	NIST17	-

4.2 Identification of the standards

Identification of any compound leads to authentication of that compound. Through identification process chemical and molecular formulas of the compound can be confirmed and based on identification purity of the compound can be checked also. Identification can be done through various processes like physical properties, chemical properties and qualitative tests of the compounds. Physical properties may include preliminary tests like color, odor, state, etc. of the compounds while in case of chemical properties assay, melting point, boiling point, spectroscopic, chromatographic, etc. methods can be used for the identification of compounds. In present research work melting point, solubility

test, FT-IR spectroscopy, GC-MS library (chromatographic method) was used for the identification of selected analytes.

4.2.1 Melting Point

- The melting point of selected analytes were determined by taking small number of standards in a capillary tube, closed at one end, and placed in a capillary melting point apparatus and the temperature at which it melts was observed. This process was repeated three times and average value was verified for all standards independently.

4.2.2 Solubility Study

- The solute and solvent give homogeneous mixture effect after dissolving into each other. The desired concentration of the standards gets dissolved into specific solvents. When the compounds are dissolved into solvents it may give better analysis. To identify the maximum solubility of the standards, various solvents have been tried. Each standard individually dissolved into each solvent to get appropriate solubility of each one of them. The solubility study of acenaphthene, anthracene, naphthalene, fluoranthene, pyrene, alpha-methyl styrene, diethyl azelate, tributyrin were performed in methanol, water, acetone, ethanol, benzene, chloroform, carbon tetrachloride, n-hexane, iso propyl alcohol, acetonitrile, formaldehyde, ethyl acetate, toluene, etc.

4.2.3 FT-IR Study

- Selected analytes were identified with FT-IR study and FT-IR spectra were recorded for confirmation by using Cary 630 model of Agilent, USA. FT-IR study for all the standards independently has been done. The study is showing spectra having information about structural data of all the standards. Stretching

and bending of the compound have been observed in the spectra. As discussed into the introduction part about the primary identification techniques of the standards FT-IR has been used to identify the compounds. The spectra obtained by FT-IR was compared with the standard spectra given by NIST.

4.2.4 GC-MS Library Search

- The instrument GC-MS of Shimadzu Corporation, Japa with model number TQ8040 having a facility of the library. The library contains information about various chemical compounds with its molecular formula, structure, name of compound, mass ratio etc. The data of this library was available by previous study performed about those compounds. All the standards were separately injected into the GC-MS instrument and the obtained chromatogram was compared with GC-MS library search with the help of NIST17 library. Also, the obtained spectra were compared to the molecular weight, name of compound and structure of each standard.

4.3 Preparations of standard solutions

4.3.1 Preparation of Auto Sampler Rinsing Solution:

- Precisely measured capacity of methanol (HPLC grade) and water (Milli-Q-Water) filled into vials and kept at room temperature.
- Rinsing solutions can be prepared through various methods. Here, rinsing solutions was prepared in three different stages.
- Stage one for rinsing was done with milli Q water followed by stage two was done by mixing methanol and water in the ratio of 50:50% v/v while stage three was done by mixing done by using only methanol as a rinsing solution.

4.3.2 Preparation of Stock Solutions:

4.3.2.1 Preparation of Acenaphthene Stock Solution-A1 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Acenaphthene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.2 Preparation of Anthracene Stock Solution-A2 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Anthracene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.3 Preparation of Naphthalene Stock Solution-A3 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Naphthalene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.4 Preparation of Fluoranthene Stock Solution-A4 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Fluoranthene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.5 Preparation of Pyrene Stock Solution-A5 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Pyrene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.6 Preparation of Alpha- Methyl Styrene Stock Solution-A6 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Alpha- Methyl Styrene was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.7 Preparation of Diethyl Azelate Stock Solution-A7 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Diethyl Azelate was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.2.7 Preparation of Tributyrin Stock Solution-A8 (SS=1.0 mg/mL)

- Accurately weighed 10 mg of Tributyrin was dissolved in 10 mL Acetone (HPLC grade) into the volumetric flask of 10mL capacity and sonicated for 10 min.

4.3.3 Preparation of individual Intermediate Solution-B for scanning (SS=500ng/mL)

- Accurately measured 100µL from individual stock solution (A1-A5) was taken in 10.0 mL volumetric flask and individually diluted up to 10 mL with Acetone to obtain final concentration of 0.5 mL of analytes and 9.5mL of acetone for group 1 and 10.1 µL of analytes and 9.9 mL of acetone for group 2. The procedure followed for the stock solution (A6-A8) and make up to the volume 10mL with acetone into the volumetric flask of 10mL capacity.

4.3.4 Preparation of Calibration Curve Solution:

Samples are accurately measured (according to the Table: 4.6) for the preparation of calibration curve each stock solution taken into the volumetric flask and diluted up to 10mL with Acetone to produce final concentration of 10 µg/mL for group 1 and 0.05 and 0.001 ng/mL for group 2. This is deliberated as a parent solution. One solution prepared for standards one to five and separate solution was prepared for standards six to eight.

Table: 4.6 Sample preparation for calibration curve

Analytes	Concentration of standard (mg/mL)	Volume taken from standard solution (mL)	Volume of diluent (mL)	Final volume (mL)	Concentration of final solution (µg/mL)
Naphthalene	1.00	0.1			10
Acenaphthene	1.00	0.1			10
Anthracene	1.00	0.1			10
Fluoranthene	1.00	0.1	9.5	10	10
Pyrene	1.00	0.1			10
	Concentration of standard (µg /mL)	**Volume taken from standard solution (µL)**	**Volume of diluent (µL)**	**Final volume (mL)**	**Concentration of final solution (ng/mL)**
Alpha- Methyl Styrene	1.00	5			0.05
Diethyl Azelate	1.00	5	9.9	10	0.05
Tributyrin	1.00	0.1			0.001

4.4 Development and optimization of Gas Chromatographic-Mass Spectroscopic method

4.4.1 Scan of Analytes in Mass Spectrometry:

- Scanning of standards in mass spectrometry was done using helium as a carrier gas.
- Scanning was done in both positive and negative ionization mode for both Precursor ion and Product ion.
- Electro Spray Ionization (ESI) was used as an ionization source.
- Different parameter like temperature and time programming, gas flow rate, column oven temperature and pressure were maintained appropriately.

4.4.2 Optimization of Chromatographic conditions:

- Based on different trials as mentioned here analytes are the development of method was done by using simultaneous estimation of the analytes. To make it easier and adoptable the analytes were divided into two groups. One group of the analytes is completely based on the materials which belongs to the rubber material category. Another group of the analytes based on the materials belongs various categories like flavouring agents, ink and dyes materials etc. also, the temperature for the separation and concentration level matters to the analytes for the separation.
- For the estimation of group one (Acenaphthene, Anthracene, Naphthalene, Fluoranthene, Pyrene) and group two (alpha-methyl styrene, diethyl azelate and tributyrin) various temperature program was set in different ratio with different time program, pressure, and flow rates.
- The trials of chromatographic conditions are given below in table: 4.7 to 4.10 for group one (naphthalene, acenaphthene, anthracene, fluoranthene and pyrene), table: 4.11 to 4.14 for group two (alpha-methyl styrene, diethyl azelate and tributyrin).

Table: 4.7 Trial 1 of chromatographic conditions for group 1

Parameters	Conditions (1)	Observation
Colum Oven Temp.	80.00 °C	No peak of selected analytes was observed.
Injection Temp.	220.00 °C	
Injection Mode	Split	
Pressure	102.70 kPa	
Total Flow	12.50 mL/min	
Column Flow	01.50 mL/min	
Linear Velocity	45.10 cm/sec	
Purge Flow	03.50 mL/min	
Oven Temp. Program	Starting with 80°c, increases to 180°c with 17°c rate and hold for 3mins, again increases to 250°c with 10°c rate and hold for 10mins, increases to 280°c with 20°c rate and hold for 15mins.	
Ion Sours Temp.	230.00°C	
Interface Temp.	280.00°C	
Total Program Time	43.00mins.	

Table: 4.8 Trial 2 of chromatographic conditions for group 1

Parameters	Conditions (2)	Observation
Colum Oven Temp.	100.00 °C	Only two analytes were observed.
Injection Temp.	250.00 °C	
Injection Mode	Split	
Pressure	123.70 kPa	
Total Flow	23.30 mL/min	
Column Flow	01.67 mL/min	

Linear Velocity	48.00 cm/sec
Purge Flow	05.00 mL/min
Oven Temp. Program	Starting with 100°c for 2min hold, increases to 120°c with 5°c rate and hold for 2mins, again increases to 200°c with 6°c rate and hold for 10mins.
Ion Sours Temp.	230.00°C
Interface Temp.	250.00°C
Total Program Time	47mins.

Table: 4.9 Trial 3 of chromatographic conditions for group 1

Parameters	Conditions (3)	Observation
Colum Oven Temp.	50.00 °C	Clear separation of two analytes were not observed. (peak resolution was not identified)
Injection Temp.	100.00 °C	
Injection Mode	Split	
Pressure	73.00 kPa	
Total Flow	11.10 mL/min	
Column Flow	01.27 mL/min	
Linear Velocity	40.80 cm/sec	
Purge Flow	03.50 mL/min	
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 100°c with 10°c rate and hold for 5mins, again increases to 150°c with 15°c rate and hold for 2mins, increases to 250°c with 15°c rate and hold for 2mins, at last increases to 280°c with 15°c rate.	
Ion Sours Temp.	250.00°C	
Interface Temp.	280.00°C	

Total Program Time	30.00mins.	

Table: 4.10 Trial 4 of chromatographic conditions for group 1

Parameters	Conditions (4)	Observation
Colum Oven Temp.	35.00 °C	All analytes were separate out clearly and method was optimized with these parameters.
Injection Temp.	250.00 °C	
Injection Mode	Splitless	
Pressure	134.30 kPa	
Total Flow	15.50 mL/min	
Column Flow	02.50 mL/min	
Linear Velocity	56.90 cm/sec	
Purge Flow	03.00 mL/min	
Oven Temp. Program	Starting with 35°c for 2min hold, increases to 250°c with 6°c rate and hold for 20mins.	
Ion Sours Temp.	250.00°C	
Interface Temp.	280.00°C	
Total Program Time	60.00mins.	

Table: 4.11 Trial 1 of chromatographic conditions for group 2

Parameters	Conditions (1)	Observation
Colum Oven Temp.	50.00°C	Proper separation of the peaks was not found.
Injection Temp.	100.00 °C	
Injection Mode	Split (ratio 10.0)	
Pressure	73.00 kPa	
Total Flow	11.10 mL/min	

Column Flow	01.27 mL/min	
Linear Velocity	40.80 cm/sec	
Purge Flow	03.50 mL/min	
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 100°c with 10°c rate and hold for 5mins, again increases to 150°c with 15°c rate and hold for 2mins, increases to 250°c with 15°c rate and hold for 2mins, at last increases to 280°c with 15°c rate.	
Ion Sours Temp.	250.00°C	
Interface Temp.	280.00°C	
Total Program Time	30.00mins.	

Table: 4.12 Trial 2 of chromatographic conditions for group 2

Parameters	Conditions (2)	Observation
Colum Oven Temp.	50.00°C	All the analytes were not separate out.
Injection Temp.	250.00°C	
Injection Mode	Split (ratio 10.0)	
Pressure	103.50 kPa	
Total Flow	24.30 mL/min	
Column Flow	01.75 mL/min	
Linear Velocity	48.00 cm/sec	
Purge Flow	05.00 mL/min	
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 200°c with 10°c rate and hold for 10mins, again increases to 300°c with 10°c rate and hold for 10mins	
Ion Sours Temp.	230.00°C	
Interface Temp.	250.00°C	
Total Program Time	50.00 mins	

Table: 4.13 Trial 3 of chromatographic conditions for group 2

Parameters	Conditions (3)	Observation
Colum Oven Temp.	50.00 °C	All analytes were separate out clearly, but temperature hold time and program time is more so further optimization was required.
Injection Temp.	250.00 °C	
Injection Mode	Split (ratio 10.0)	
Pressure	103.50 kPa	
Total Flow	24.30 mL/min	
Column Flow	01.75 mL/min	
Linear Velocity	48.00 cm/sec	
Purge Flow	05.00 mL/min	
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 100°c with 10°c rate and hold for 5mins again increases to 150°c with 10°c rate and hold for 10 mins, at last increases to 200°c with 10°c and hold for 10 mins	
Ion Sours Temp.	250.00°C	
Interface Temp.	280.00°C	
Total Program Time	37.00 mins.	

Table: 4.14 Trial 4 of chromatographic conditions for group 2

Parameters	Conditions (3)	Observation
Colum Oven Temp.	50.00°C	All analytes were separate out clearly.
Injection Temp.	250.00°C	
Injection Mode	Split (ratio 10.0)	
Pressure	103.50 kPa	
Total Flow	24.30 mL/min	
Column Flow	01.75 mL/min	
Linear Velocity	48.00 cm/sec	

Purge Flow	05.00 mL/min
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 100°c with 10°c rate and hold for 5mins again increases to 150°c with 10°c rate and hold for 5mins, at last increases to 200°c with 10°c and hold for 5 mins
Ion Sours Temp.	250.00°C
Interface Temp.	280.00°C
Total Program Time	32.00 mins.

4.5 Validation of Developed Method According to ICH Q2 (R1) guideline

Validation of an analytical method assure that the process is generating the results which leads to the acceptance criteria consistently. There are various guidelines according to the process for the validation. In current research validation was done according to the ICH Q2 (R1) guidelines. Here validation includes the parameters like quality, sample matrix, reliability, concentration level, expected interface, etc. through linearity, precision, detection limit, accuracy, quantitation limits, etc.

4.5.1 Linearity and Range (Calibration curve):

- Multi-level calibration (three or more levels) is preferred. Calibration levels are selected and was prepared according to the procedure described in table: 4.6 Then standards are injected into the GC-MS/MS.
- The calibration curve should not typically be forced through the origin without a valid reason, and a suitable calibration function must be utilised. To make sure the fit is adequate within the concentration range of the leachables found, the calibration function's fit must be displayed and examined visually and/or by computing correlation coefficients.

- An alternate calibration function must be employed if individual analytes in the relevant region depart from the calibration curve by more than ±20%. Instead of using linear regression, weighted linear regression (1/x2) is generally advised.
- The concentration of the analytes was calculated from the Area v/s Concentration by regression equations:

$$y = mx \pm c$$

Were,

y = peak area of individual standards Acenaphthene, Anthracene, Naphthalene, Fluoranthene, Pyrene.

m = slope of the calibration curve

x = concentration of individual standards Acenaphthene, Anthracene, Naphthalene, Fluoranthene, Pyrene.

C= Intercept

4.5.2 Accuracy:

- **Accuracy** was evaluated by measuring %mean accuracy at each concentration levels of calibration curve standards. Accuracy of each level was within 98%-102%.
- **Acceptance criteria:** %CV of accuracy and precision was within ≤2%.
- Equation to find the accuracy is given below.

$$\% \text{ Nominal} = \frac{\text{Concentration found}}{\text{Nominal concentration}} \times 100$$

4.5.3 Precision:

- **Precision** was performed by using six replicates of any on concentration from standards of linearity range. Precision was calculated by measuring %CV of each concentration levels.
- **Acceptance criteria:** %CV of accuracy and precision was within ≤2%.
- Equation to find the precision is given below.

$$\% \text{ CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Where,

%CV = Coefficient of Variation

4.5.4 Robustness:

- Robustness of method was performed by one linearity batch performed and batch was analysed by different chromatographic parameter.

4.5.5 Limit of Detection (LOD) and Limit of Quantification (LOQ):

- **LODs** were estimated by analysing individual standard samples which can be detected but not necessarily quantitated as an exact value.

LOD

$$\text{LOD} = \frac{3.3 \times \text{SD}}{\text{Slope}}$$

Where,

LOD= Limit of Detection

SD= Standard Deviation

Slope= Slope of Calibration Curve

- **LOQs** were determined with suitable precision and accuracy. The minimum concentration that provides suitable accuracy and precision.

LOQ

$$\text{LOQ} = \frac{10 \times \text{SD}}{\text{Slope}}$$

Where,

LOD= Limit of Quantification

SD= Standard Deviation

Slope= Slope of Calibration Curve

4.5.6 Specificity:

- The components which may be expected to be present, in presence of them ability to assess clearly the analyte in the sample is represent the term specificity. To check whether the method is specific to compound or not solvents in which the analytes were dissolved may be used.

4.6 Application of the developed and validated method for identification of extractables and leachables in selected pharmaceutical formulations

- Various pharmaceutical formulations of parenteral and ophthalmic products were collected for the identification of extractables leachables from the local market of Rajkot city.
- After the identification of extractables and leachables in pharmaceutical formulations, time and temperature assisted extraction techniques were used.
- The developed method may be used to quantify the selected extractables and leachables from different pharmaceutical products procured from market.
- Selected pharmaceutical formulations for the application of the developed method are listed below.

Table: 4.15 List of Pharmaceutical Formulations Selected for the Study

Sr no.	Name of Formulation	Type of Formulation
1.	Carboxy methyl cellulose	Eye drops
2.	Chlorbutol, benzocaine and paradichlorobenzene	Ear drops
3.	Sterile water	Sterile water for injection
4.	Normal Saline	Intravenous Solution
5.	Ciprofloxacin	Intravenous Infusion
6.	25D (dextrose)	Intravenous Solution
7.	Metronidazole	Intravenous Infusion

4.7 Extraction Techniques

Extraction is process which performed to isolate the targeted compounds from the product. Through the extraction process extreme targeted compounds can be recovered and unwanted materials can be avoided. Amongst various extraction techniques as mentioned in the introduction 1.4.2 part like Soxhlet extraction, reflux extraction, liquid liquid extraction, pH extraction, sunlight extraction, solid phase extraction etc., here in this present work based on literature following extraction techniques were applied for the study. Extraction techniques like liquid-liquid extraction, reflux condensation, pH buffer solution and sunlight were tried for the present research work.

Table: 4.16 Extraction techniques with conditions

Sr no.	Extraction Techniques	Solvent Used	Conditions Applied
1	Liquid-Liquid Extraction	Dichloromethane	24 Hrs. at room temperature
2	Reflux Condensation	Dichloromethane Isopropyl Alcohol n-Hexane	6 Hrs. at 55˚c (Water Bath)
3	pH	Ammonia Buffer: 9.5pH Phosphate Buffer: 2.5pH	3-4 Days at 50-70˚c (Hot Air Oven)
4	Sunlight	-	About 2 Months

4.7.1 Extraction Process

4.7.1.1 Liquid-Liquid Extraction: 50:50 ratio of the solvent and formulation i.e.10mL of solvent (DCM) added to 10mL of the formulation individually. The mixture was kept at room temperature for 24 hours with occasional shaking. At last, the separate layer was collected and evaporated completely. The evaporated components were recollected in solvent by vertex mixer.

4.7.1.2 Reflux Condensation: A round bottom flask charged with DCM, IPA and n-Hexane individually, were attached to the heat and on another end, vapor meets ice cold water to recycle the solvent vapor and formulation, cooled vapor converted into liquid state again and fall into mixture repeatedly. The reflux assembly was set at a temperature of approximately 55˚c, aimed at 6 hours constantly for each pharmaceutical formulation in each solvent.

4.7.1.3 pH: Two different solutions of two different pH i.e. of pH 2.5 (Phosphate buffer- mixture of potassium hydrogen phosphate, water and HCl) and pH 9.5 (Ammonia buffer- mixture of ammonium chloride, water and 10M ammonia) were filled into the packaging of the formulation as available. The pH filled

formulations were kept into hot air oven for 3-4 days at 50-70˚c temperature with continuous observation.

4.7.1.4 Sunlight: All the formulations with packaging material were kept into direct sunlight. Due to the heat and rays of sunlight the formulations reacted with the packaging materials and leached into the product. The study of direct sunlight is intended for 2 months (with continuous observation at 15 days interval).

4.8 *Invitro* Toxicity Study

- Biological evaluation and screening test using cells of tissue for the observation of various activities of cell like morphological changes, modification in the reproduction etc. by medical devices is done *in vitro* for the cytotoxicity testing. From the study potentiality of the compound to produce cell death or the effect of the cell on the viability of cells can be identified. Induced chemicals may show the effect of potential leachables on the genotoxicity, inflammation and stress of oxidative condition of the cells. To observe the viability of the cells before giving treatment of the potential material and after giving treatment with the potential material, also percentage of the viability or cell death testing is performed by using cytotoxicity testing.

- Cell Line: Originating from primary cultures that have a finite lifespan, the population exhibits a certain level of genotypic and phenotypic uniformity because of the passage of cells with the highest growth capability.

- Using animal cell culture, *in vitro* toxicity testing examines how novel medications, cosmetics, and chemicals affect the growth and survival of various cell types, particularly liver and kidney cells.

- Cultured animal cells are also used to determine the maximum permissible dosage of new drug. MTT assay has been performed for the toxicity testing.
- MTT Assay: MTT is [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium] reduced by metabolically active cells to purple formazan. The absorbance of the colored solution is then quantified spectrophotometrically. An increase or decrease in cell number results concomitant change in the amount of MTT formazan formed and consequently in the absorbance.

Table: 4.17 List of materials for cell line study

Materials	Ingredients	Manufacturer/ Supplier
Cell Line	HEK-293 (is a cell line exhibiting epithelial morphology that was isolated from the kidney of a human embryo) MCF-7 (is a human breast cancer cell line)	NCCS (National Centre For Cell Science), Maharashtra, India
Cell Counting	Trypan Blue Dye	Invitrogen 3FL-Thermofisher Scientific, Massachusetts, US
Media	DMEM (Dulbecco's Modified Eagle's Medium) High Glucose Media: 4.5g/l Glucose, L-glutamine, 3.7g/l Sodium Bicarbonate and Sodium Pyruvate	HIMEDIA, Maharashtra, India
Growth Supplement	10% FBS (Fetal Bovine Serum)	HIMEDIA, Maharashtra, India
Antibiotic	2.5 µg/mL- Gentamicin	HIMEDIA, Maharashtra, India
Contamination Prevention (from yeast and multicellular fungi)	2.5µg/mL- Amphotericin B	HIMEDIA Maharashtra, India
MTT Solution	12mM (5mg/mL) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium	-

	PBS (Phosphate Buffered Saline): 0.2g Potassium Chloride, 0.2g Potassium Phosphate Monobasic, 8g Sodium Chloride, 1.15g Sodium Phosphate Dibasic (Adjusted at pH 7.3 with 1M NaOH)	-
Dimethyl sulfoxide (DMSO)	AR Grade	Rankem, India

4.8.2 Equipment, Instrument, and other Accessories

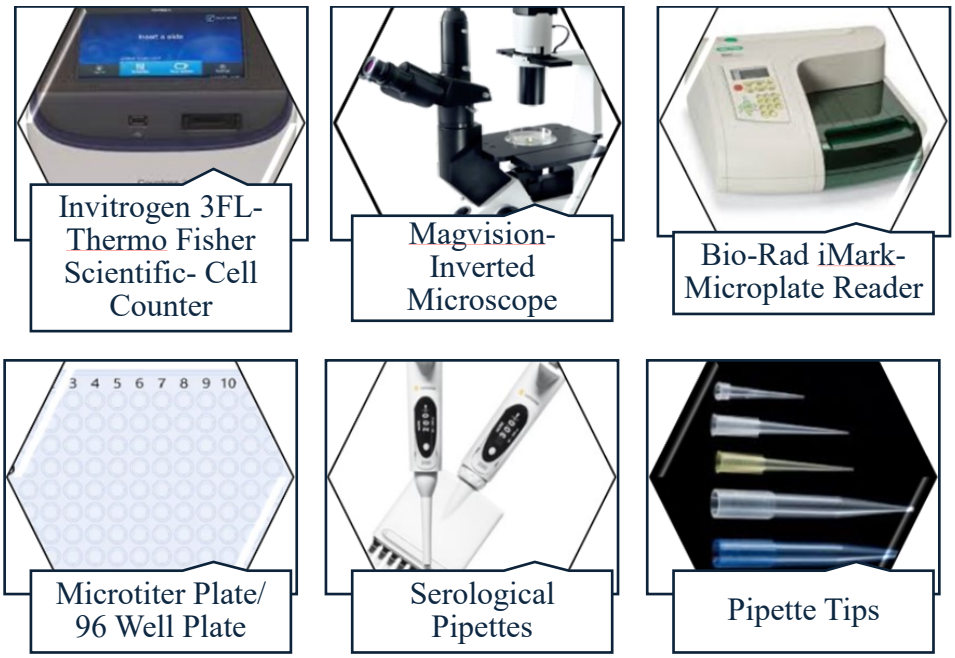


Figure: 4.1 Equipments and other materials used for cell line study

4.8.3 Sample Preparation

- The sample for cell line study was prepared after some trials of sample preparation, that trials are described as below.
- Preparation of standard stock solution(1000µg/mL) Trial-1: accurately weighed 1 mg of individual standard were dissolved in 1 mL of DMSO and vertex till complete dissolution.

- Preparation of standard stock solution(1000µg/mL) Trial-2: accurately weighed 1 mg of individual standard were dissolved in 1 mL of Acetone and vertex till complete dissolution.
- Preparation of standard stock solution(1000µg/mL) Trial-3: accurately weighed 100 mg of individual standard were dissolved in 1 mL of DMSO and vertex till complete dissolution.
    - Withdraw 0.1mL from the solution and dissolved in MiliQ water to make up to 1mL volume.

4.8.4 Methodology for cell line study

- **Passaging:** Passaging or spitting of the cells is done after counting the number of cells through the cell counter. Calculated cells are divided equally to each well of plate and add nutrients to each well as well. Subsequently add the cell and nutrients filled well plate into CO_2 incubator at 37±5˚c for 24 hours.
- **Sample Application:** Sample of selected analytes and media for the cell were prepared accordingly and added to the first line of well. There after adding half concentration of the first line to the next line in accordance with serial dilution up to 6 concentration level. Again, keep the well plate into CO_2 incubator at 37±5˚c for 24 hours.
- **MTT Assay:** In this process MTT solution was applied to the wells and incubated under CO_2 incubator at 37±5˚c for 3 to 4 hours. At the end add DMSO solvent to each well and quantify the cells with microplate reader.

Chapter- 5

Results and Discussions

5.1 FT-IR Study Results

- Fourier Transform Infrared Spectroscopy (FTIR) is a technique that provides high-resolution analysis of a sample's infrared absorption across a wide spectral range. An FT-IR spectrum, also known as a Fourier Transform Infrared spectrum, is a graphical representation of how much infrared radiation a sample absorbs at different wavelengths. It functions as a "molecular fingerprint" that can be used to identify specific chemical compounds based on the unique vibrational modes of their bonds, which are shown as peaks at characteristic wavenumber positions on the spectrum.
- Identification of all the standards independently has been done with FT-IR study. The spectra obtained by FT-IR was compared with the standard spectra given by NIST.
- FT-IR spectra are given below for all analytes.

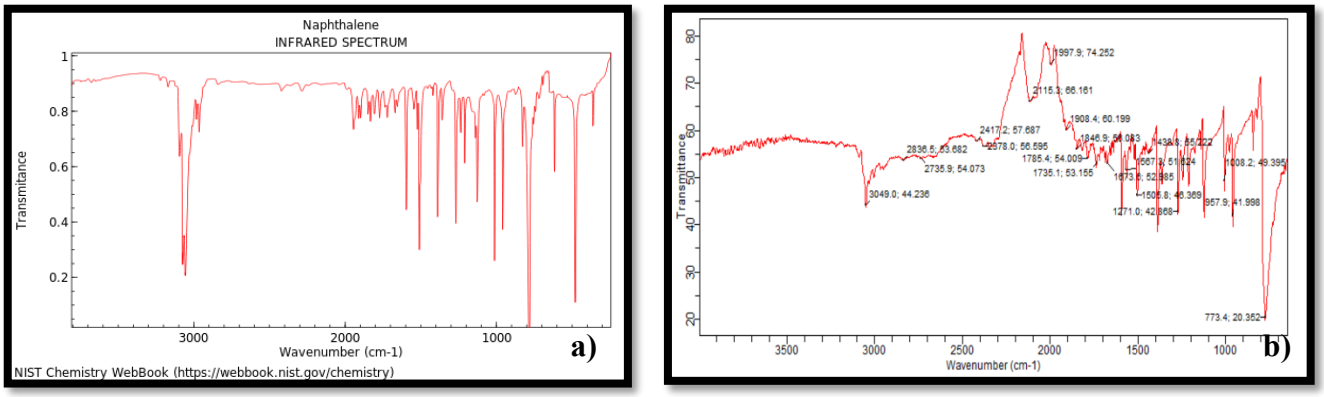


Figure: 5.1 FT-IR spectra of Naphthalene a) standard b) test

- The terahertz spectrum of room-temperature naphthalene was measured using the Fourier Transform Infrared method. Six absorption bands were visible in the 5–15 THz range for naphthalene. The bands appear at 5.80 and 6.37 THz. The absorption peaks are a result of the movement of the molecule as a whole. The KBr pellet method is used to record the spectrum. The C-H stretching of naphthalene is attributed to the spectrum's high peak at 3050.18 cm−1. Other peaks in the spectrum, such as those attributed to naphthalene's ring skeletal vibration, are also visible.
- As per the figure 5.1 the spectra of standard naphthalene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

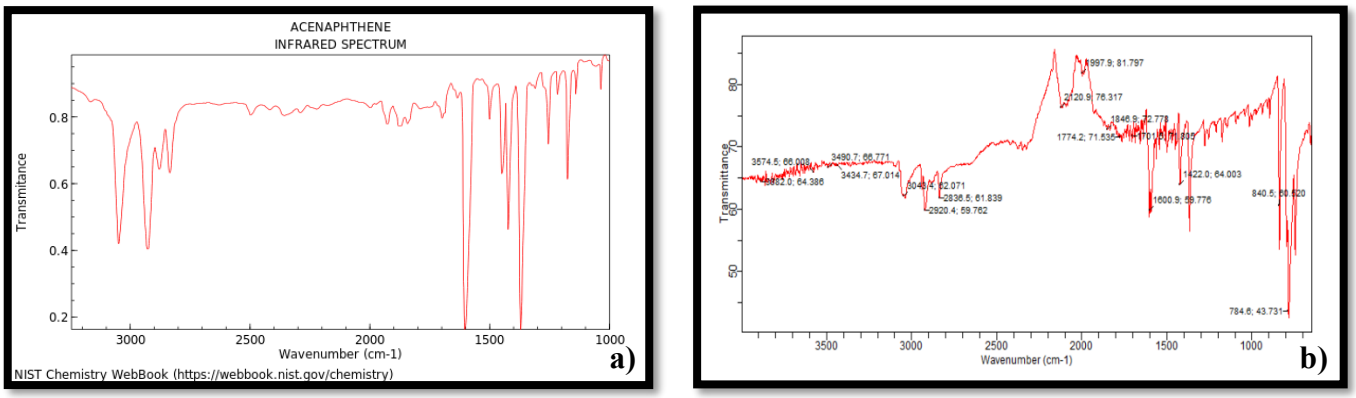


Figure: 5.2 FT-IR spectra of Acenaphthene a) standard b) test

- The presence of the aromatic ring system within the acenaphthene molecule is indicated by the most prominent peaks in the fingerprint region (below 1500 cm^-1) of the FTIR spectrum. These peaks correspond to the vibrational modes of the aromatic rings, including C-C stretching vibrations, with characteristic peaks around 1600 cm^-1, 1450 cm^-1, and 1200 cm^-1. Additionally, the C-H stretching vibrations of the aromatic hydrogens will show absorption bands in the region between 3000-3100 cm^-1.

- Strong absorption bands between 3000 and 3100 cm^-1 are caused by the aromatic hydrogen atoms' stretching vibrations. Significant peaks that show the stretching vibrations inside the aromatic rings are located at 1600 cm^-1, 1450 cm^-1, and 1200 cm^-1. Because of the molecule's complicated vibrational modes, the fingerprint region below 1500 cm^-1 will exhibit the most distinctive characteristics of the acenaphthene spectrum.
- As per the figure 5.2 the spectra of standard acenaphthene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

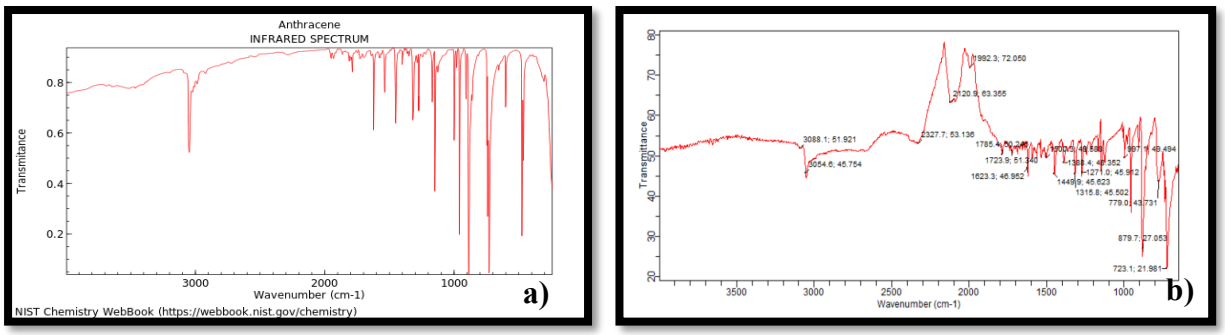


Figure: 5.3 FT-IR spectra of Anthracene a) standard b) test

- Because of its distinct "fingerprint" pattern in the infrared spectrum, anthracene can be identified by its most noticeable peaks, which usually appear in the fingerprint region (below 1500 cm-1) with notable absorption bands around 880 cm-1, 750 cm-1, and 1260 cm-1, which correspond to the distinctive vibrational modes of the aromatic ring system within the anthracene molecule. The most prominent peaks are usually observed around 880 cm-1 (out-of-plane bending of C-H bonds), 750 cm-1 (in-plane bending of C-H bonds), and 1260 cm-1 (C-C stretching vibrations within the aromatic rings). Most significant absorption bands for anthracene are located in the fingerprint region below 1500 cm-1, which allows for identification based on the unique pattern of peaks.

- As per the figure 5.3 the spectra of standard anthracene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

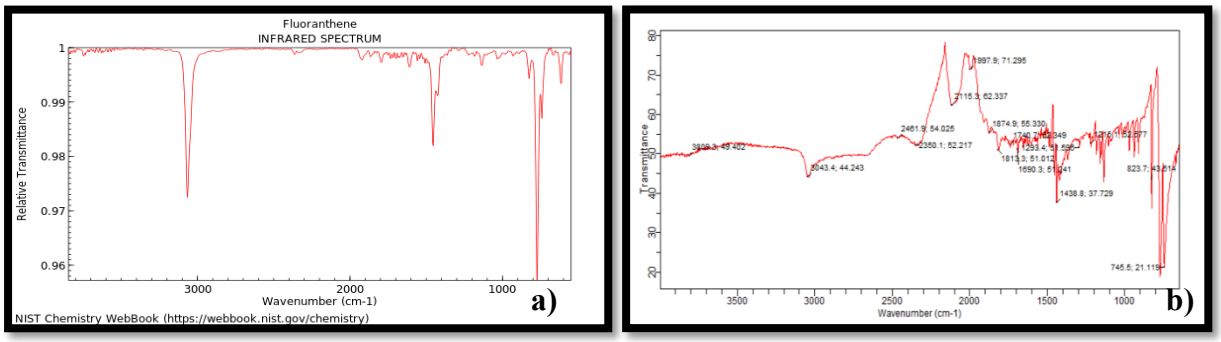


Figure: 5.4 FT-IR spectra of Fluoranthene a) standard b) test

- The idiosyncratic absorbance from 1650 to 1420 cm−1 that corresponds to the C=C aromatic stretches was visible in the FT IR spectrum. Between 900 and 690 cm−1, the =C–H aromatic out-of-plane banding is present.
- As per the figure 5.4 the spectra of standard fluoranthene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

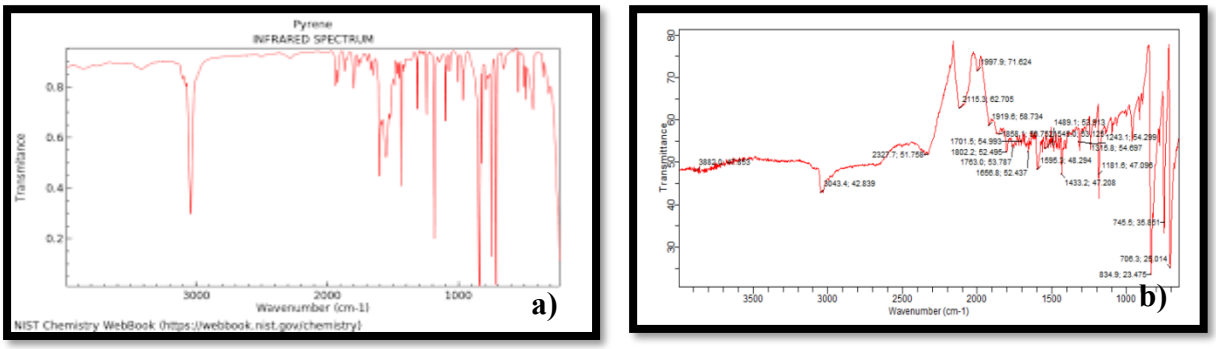


Figure: 5.5 FT-IR spectra of Pyrene a) standard b) test

- The characteristic C-H out-of-plane bending vibrations of the aromatic ring system are responsible for the most noticeable peak in an FTIR spectrum of pyrene, which appears at about 844 cm-1. Other notable peaks can be seen in

the 3000–3100 cm-1 region for C-H stretching vibrations and around 1600 cm-1 for C=C stretching vibrations within the aromatic rings. This peak, which is the strongest in the spectrum, is used as a diagnostic indicator to identify pyrene. Numerous peaks in this region are indicative of the aromatic C-H bonds' stretching vibrations. The stretching vibrations of the aromatic C=C bonds are responsible for the peaks in this area.

- As per the figure 5.5 the spectra of standard pyrene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

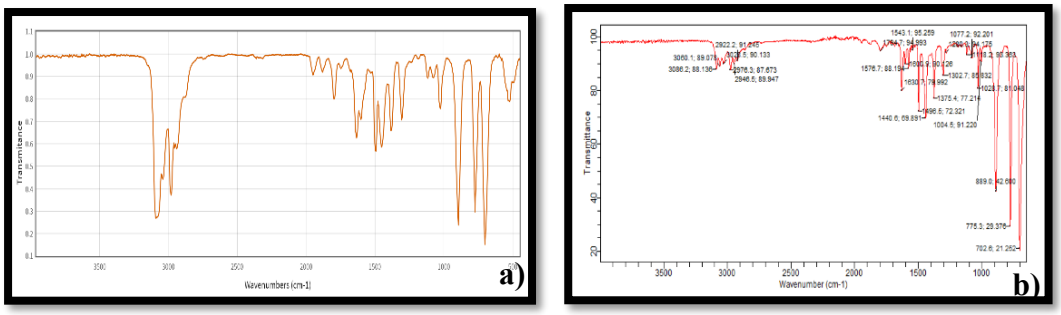


Figure: 5.6 FT-IR spectra of Alpha-methyl Styrene a) standard b) test

- The stretching vibrations of the aromatic hydrogen atoms on the benzene ring are represented by a prominent absorption band at about 3020–3080 cm-1. a moderate peak at about 1620 cm-1, which is associated with the vinyl group's carbon-carbon double bond's stretching vibration. bands at about 1600 cm-1 and 1490 cm-1, which are ascribed to the aromatic ring's distinctive stretching vibrations. Peaks around 1370 cm-1 and 1450 cm-1, representing the bending vibrations of the methyl group attached to the double bond. A band around 700-750 cm-1, arising from the out-of-plane bending vibrations of the aromatic hydrogen atoms.

- As per the figure 5.6 the spectra of standard alpha-methyl styrene given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

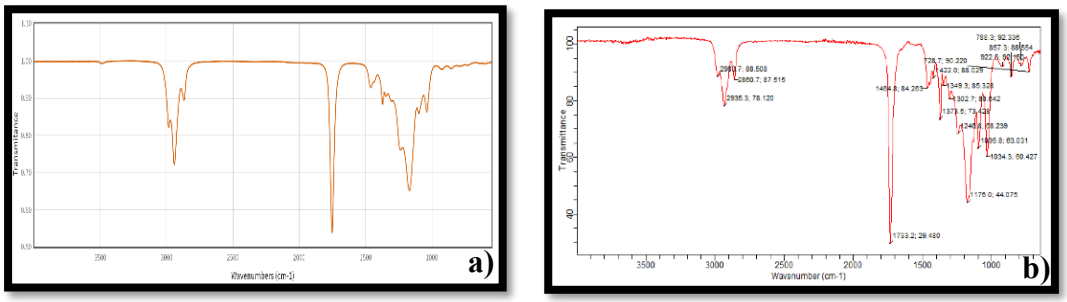


Figure: 5.7 FT-IR spectra of Diethyl Azelate a) standard b) test

- The presence of a long aliphatic chain with ester functional groups at each end is indicated by the key characteristic peaks in an FTIR spectrum of Diethyl Azelate, which are located around 1735 cm-1 (strong, carbonyl stretching), 2930 cm-1 (medium, methylene stretching), 1460 cm-1 (medium, methylene bending), 1150 cm-1 (medium, ester C-O stretching), and a series of smaller peaks around 720 cm-1 (methylene rocking). The carbonyl group's (C=O) stretching vibration within the ester functional group is represented by the strongest peak in the spectrum. Due to the stretching vibrations of the CH2 groups in the long carbon chain, a broad peak was seen; if CH3 groups were present as impurities, the peak might have been smaller. The bending vibrations of the chain's methylene groups are what cause this peak. a peak of medium intensity that represents the ester group's C-O bond's stretching vibration. a long, linear chain of smaller peaks in the fingerprint region that represent the methylene groups' rocking motions.
- As per the figure 5.7 the spectra of standard diethyl azelate given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

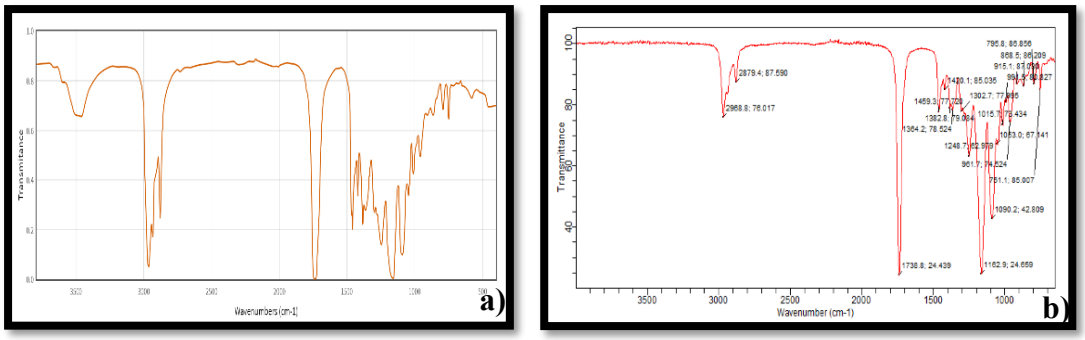


Figure: 5.8 FT-IR spectra of Tributyrin a) standard b) test

- A projecting peak at about 1740 cm-1, which stands for the carbonyl group in the triglyceride molecule's ester bond. The stretching vibrations of the methylene groups in the butyric acid chains are shown by the strong peaks between 2900 and 2850 cm-1. a noticeable peak at approximately 1160 cm-1, which is ascribed to the ester functional group's C-O single bond. a peak of medium strength at about 1460 cm-1, which is associated with the methylene groups' bending vibrations.
- As per the figure 5.8 the spectra of standard tributyrin given by NIST is matching with the test spectra of naphthalene. From the spectra it may considered that the standard analyte is fulfilling the requirements.

5.2 Optimized Chromatographic Conditions

- The process of optimising chromatographic settings includes determining the ideal values for the parameters that influence the component separation of a sample. These variables include temperature, flow rate, mobile phase, and column. Following are the steps for the optimization of the chromatographical conditions. Establish analytical goals: Choose the goals you hope to accomplish with your experiment. Choose the appropriate column: Select a column according to the size, complexity, and type of the sample. Adjust the temperature to your preference by raising or lowering it. Adjust the carrier gas's flow rate to

maximise it. Assess sample preparation by experimenting with various methods. To get the better resolution and separation of the analyte's optimization should be done. Here the method development was divided into two parts to overcome the optimization parameters.

- The estimation of leachables was done by various chromatographic conditions trials. Trails were done to get optimised method for the separation of selected analytes. Here, two methods were developed where one is for five and another one is for three different analytes. By doing changes of temperature program, flow rate, pressures the method can be optimised.
- The optimised chromatographic conditions for both methods are given in table 5.1 and 5.2 respectively.

Table: 5.1 Optimized chromatographic conditions for group 1

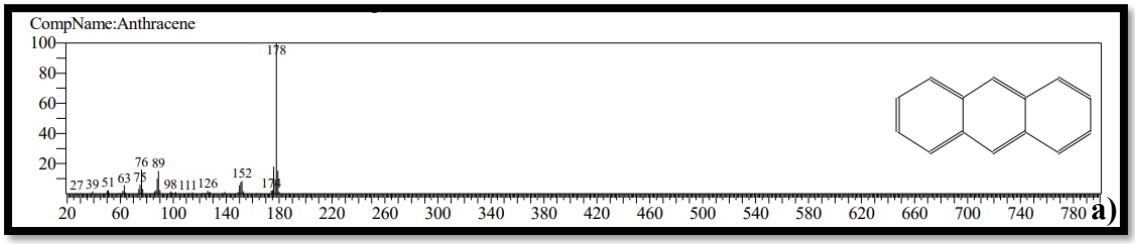
Parameters	Conditions
Colum Oven Temp.	35.0 °C
Injection Temp.	250.00 °C
Injection Mode	Splitless
Pressure	134.3 kPa
Total Flow	15.5 mL/min
Column Flow	2.50 mL/min
Linear Velocity	56.9 cm/sec
Purge Flow	3 mL/min
Oven Temp. Program	Starting with 35°c for 2min hold, increases to 250°c with 6°c rate and hold for 20mins.
Ion Sours Temp.	250.00°C
Interface Temp.	280.00°C
Retention Time	
Naphthalene	14.34±0.2 minutes
Acenaphthene	21.41±0.2 minutes
Anthracene	27.72±0.2 minutes
Fluoranthene	32.43±0.2 minutes
Pyrene	33.19±0.2 minutes
Total Program Time	60 mins.
System Controllers	
Washing volumes	8 µL
Plunger Speed (Suction) (Injection)	High
Pumping Times	5
Pre-Rinse with Solvent	6
Rinse with Sample	3
Post-Rinse with Solvent	6

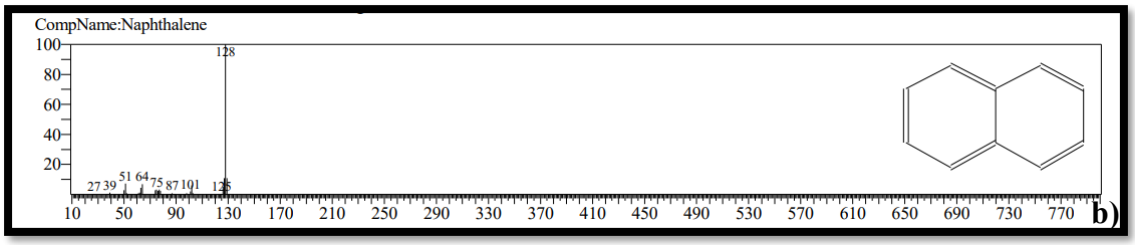
Table: 5.2 Optimized chromatographic conditions for group 2

Parameters	Conditions
Colum Oven Temp.	50.0 °C
Injection Temp.	250.00 °C
Injection Mode	Split (ratio 10.0)
Pressure	103.5 kPa
Total Flow	24.3 mL/min
Column Flow	1.75 mL/min
Linear Velocity	48.0 cm/sec
Purge Flow	5.0 mL/min
Oven Temp. Program	Starting with 50°c for 2min hold, increases to 100°c with 10°c rate and hold for 5mins again increases to 150°c with 10°c rate and hold for 5mins, at last increases to 200°c with 10°c and hold for 5 mins
Ion Sours Temp.	250.00°C
Interface Temp.	280.00°C
Retention Time	
Alpha-Methyl Styrene	5.72±0.2 minutes
Diethyl Styrene	23.77±0.2 minutes
Tributyrin	26.69±0.2 minutes
Total Program Time	34 mins.
System Controllers	
Washing volumes	10 µL
Plunger Speed (Suction) (Injection)	High
Pumping Times	5
Pre-Rinse with Solvent	6
Rinse with Sample	3
Post-Rinse with Solvent	6

5.3 Mass Spectra

- As of their high sensitivity and capacity to analyse complex mixtures at the molecular level, mass spectra are an essential tool in a variety of fields, including chemistry, biology, medicine, and forensics. They are useful because they offer a detailed analysis of a molecule's mass-to-charge ratio (m/z), which enables scientists to identify unknown compounds, quantify known substances, and determine their chemical structures. Mass spectrometry may detect new chemicals by precisely measuring a molecule's m/z and comparing the measured value to a database of known compounds. By displaying the many components of a molecule that fragment during ionisation, fragmentation patterns in a mass spectrum can offer information about the chemical structure of the molecule. Mass spectrometry makes it possible to quantitatively analyse a variety of substances by precisely determining the concentration of a particular molecule in a sample. Mass spectrometers are useful for analysing trace levels of chemicals since they can identify incredibly small amounts of analytes.





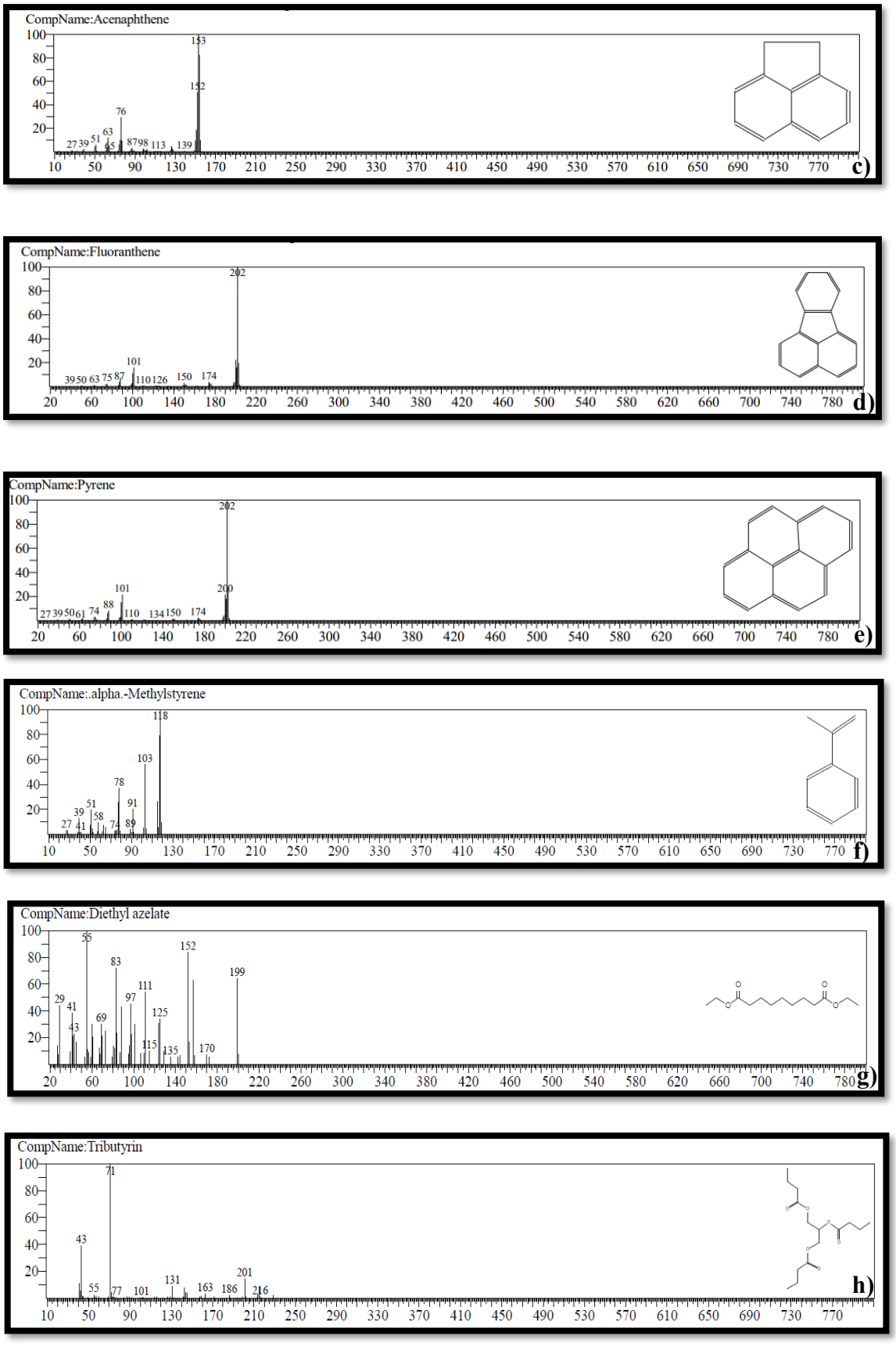


Figure: 5.9 Mass Spectra of all Standards a) Naphthalene, b) Acenaphthene, c) Anthracene, d) Fluoranthene, e) Pyrene, f) Alpha-methyl Styrene, g) Diethyl Azelate, h) Tributyrin

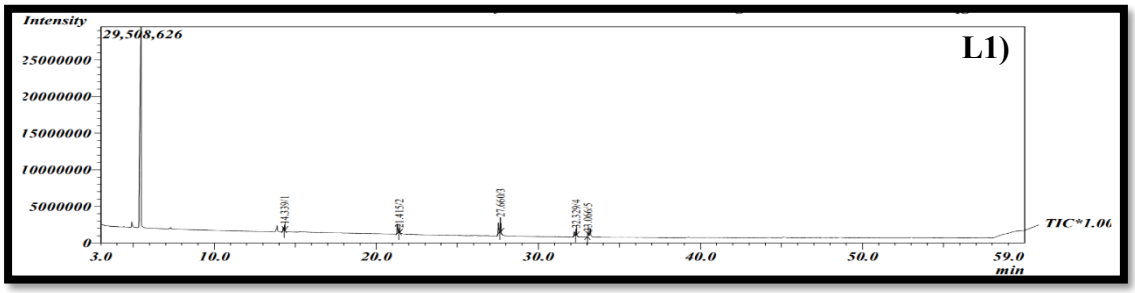
5.4 Validation of Developed Method

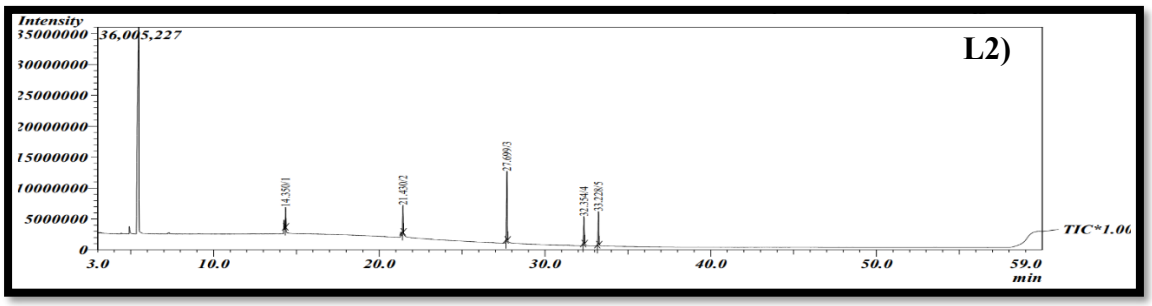
- Essentially demonstrating that the method generates reliable data when analysing samples, analytical method validation is essential for regulatory compliance, product safety, and reliable decision-making across a variety of industries, including pharmaceuticals, food, and environmental monitoring. It guarantees that an analytical method is accurate, precise, reliable, and appropriate for its intended purpose. Validation reduces errors and gives confidence in the data provided by ensuring that the analytical procedure consistently yields accurate and reproducible results. Strict regulatory requirements in many businesses, particularly the pharmaceutical sector, demand the validation of analytical techniques used for product testing and quality control. Labs can find and fix possible problems that can compromise the precision and accuracy of their analysis by validating their methods, which will ultimately improve the quality of the final output. Validation gives the analytical process a recorded scientific foundation, enabling open assessment and explanation of the findings. Once a method has been verified, it can be safely used to other labs or manufacturing locations and still yield consistent findings.
- As per the guideline ICH Q2(R1) the analytical method validation has been performed by using parameters like specificity, linearity and range, accuracy, precision, limit of detection, limit of quantification, robustness and system suitability parameters.

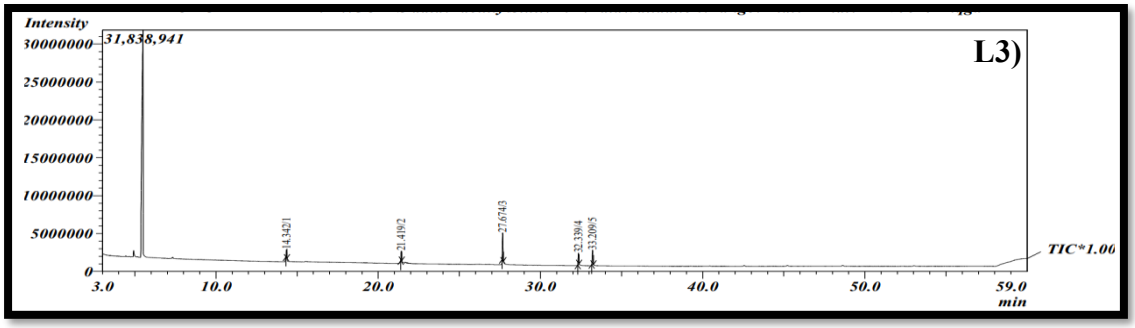
5.4.1 Linearity and Range:

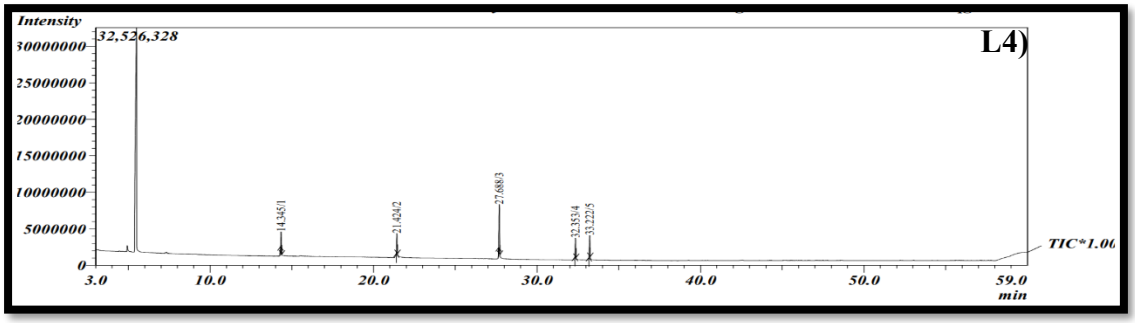
- Three times calibration curve were used to establish Linearity. Calibration curve was found to be precise and accurate for all leachables. The range of group 1

(naphthalene, acenaphthene, anthracene, fluoranthene and pyrene) was established from 30µg/mL to 10µg/mL and for group 2 alpha-methyl styrene, diethyl azelate and tributyrin it was found from 250-50 ng/mL, 250-50 ng/mL and 50-10 ng/mL respectively. The correlation coefficient was greater than or equal to 0.990 for all leachables and concentration at each calibration level was back calculated from the calibration curves. The results obtained are shown in the table were within the acceptance criteria that is R2 ≥ 0.990.









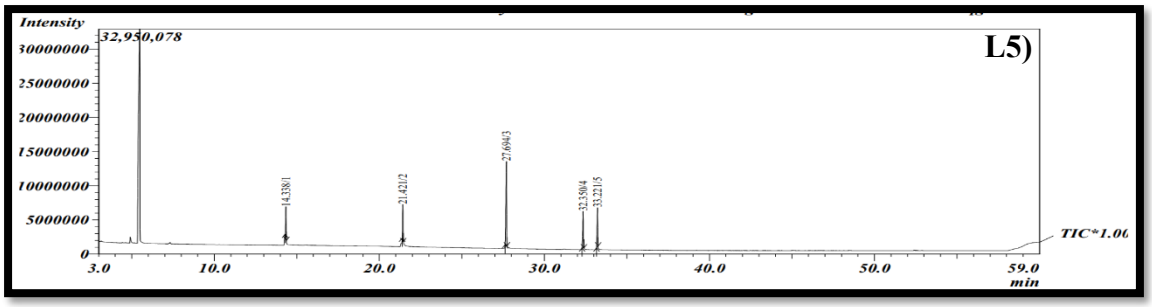


Figure: 5.10 Chromatogram of Linearity for group 1 (naphthalene, acenaphthene, anthracene, fluoranthene and pyrene) (L1- L5 different concentration level)

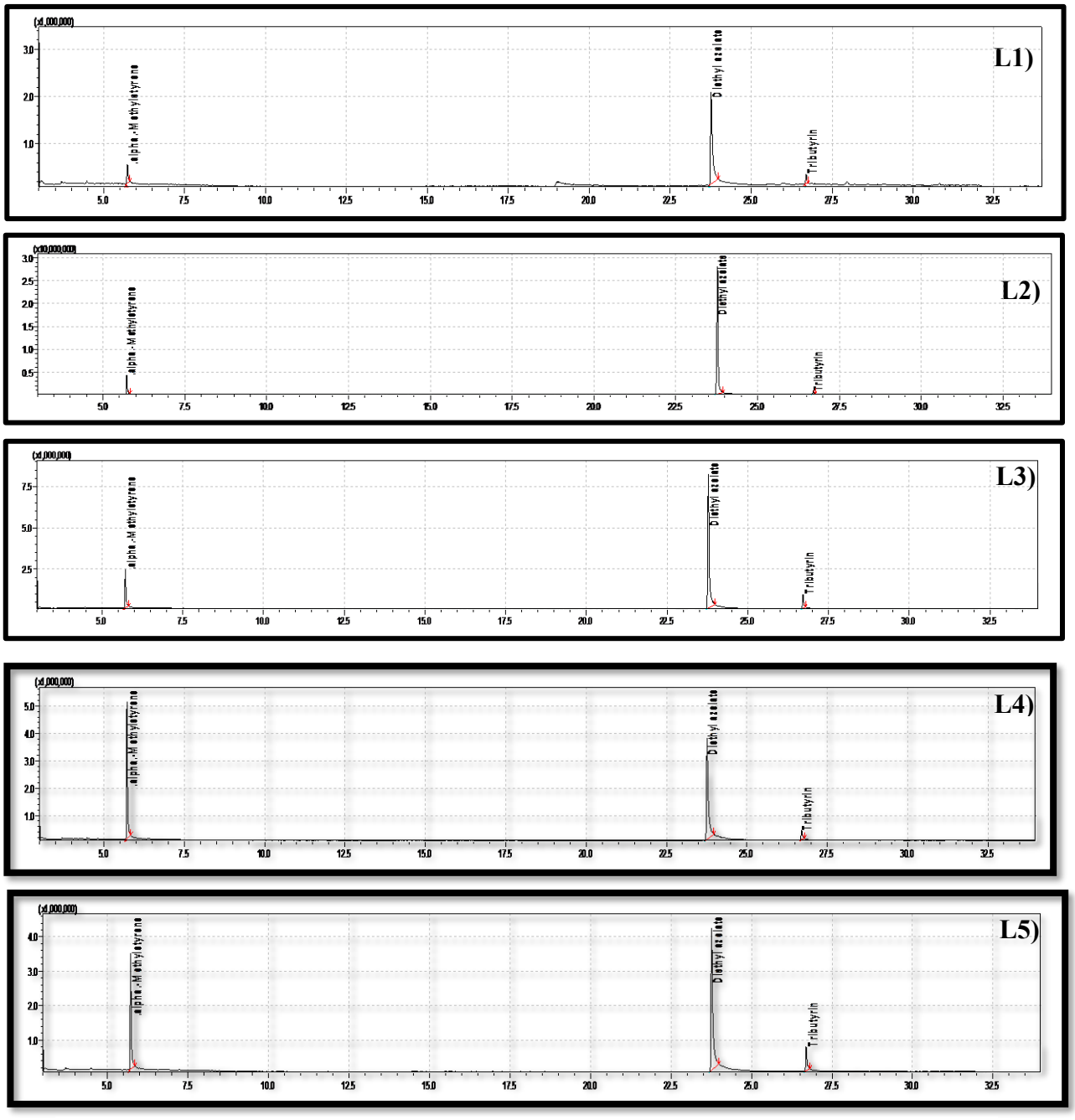


Figure 5.11 Chromatogram of Linearity for group 2 (alpha-methyl styrene, diethyl azelate, tributyrin) (L1- L5 different concentration level)

A) Linearity of Naphthalene

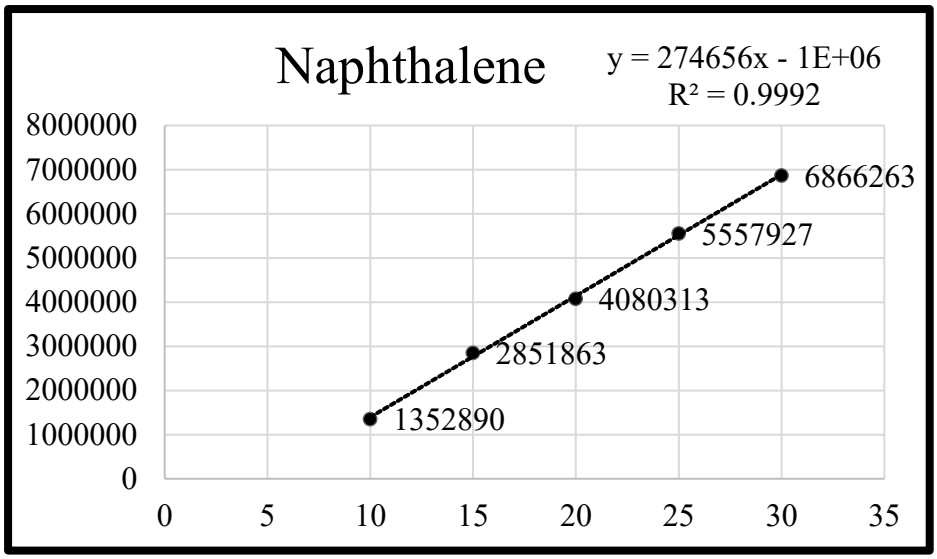


Figure: 5.12 Linearity curve of Naphthalene

Table: 5.3 Results of Linearity for Naphthalene

Linearity	Slope	Intercept	R^2
L-1	274656	1E+06	0.99
L-2	278248	1E+06	0.99
L-3	258989	1E+06	0.99
Mean	270631	1E+06	0.99

Table: 5.4 Back Calculated Values of Naphthalene

Actual Concentration (µg/mL)	**10**	**15**	**20**	**25**	**30**
Obtained Concentration (µg/mL)	8.56	14.02	18.49	23.87	28.64
	8.55	14.58	18.99	22.99	27.61
	9.15	14.64	20.03	25.08	30.78
Avg.	8.75	14.41	19.17	23.98	29.01
SD	0.34	0.34	0.78	1.04	1.61
%CV	3.90	2.37	4.09	4.37	5.58

B) Linearity of Acenaphthene

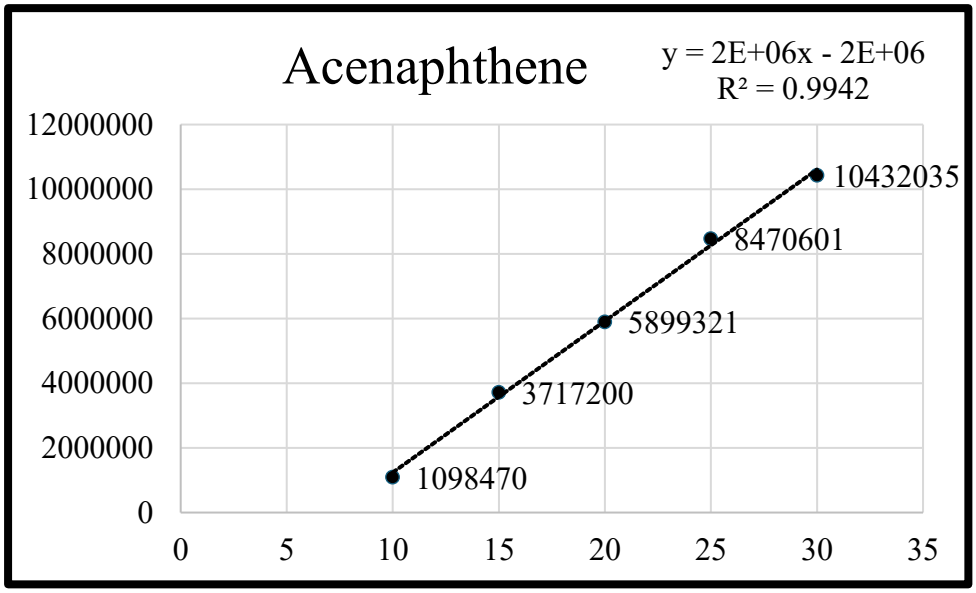


Figure: 5.13 Linearity curve of Acenaphthene

Table: 5.5 Results of Linearity for Acenaphthene

Linearity	Slope	Intercept	R^2
L-1	468411	3E+06	0.99
L-2	470268	3E+06	0.99
L-3	475268	4E+06	0.99
Mean	471315	3.33E+06	0.99

Table: 5.6 Back Calculated Values of Acenaphthene

Actual Concentration (µg/mL)	**10**	**15**	**20**	**25**	**30**
Obtained Concentration (µg/mL)	8.74	14.34	18.99	24.48	28.67
	8.83	14.23	19.05	24.16	28.86
	10.54	15.05	20.47	26.50	30.26
Avg.	9.37	14.84	19.51	25.05	29.27
SD	1.01	0.96	0.83	1.26	0.86
%CV	10.82	6.48	4.27	5.06	2.97

C) Linearity of Anthracene

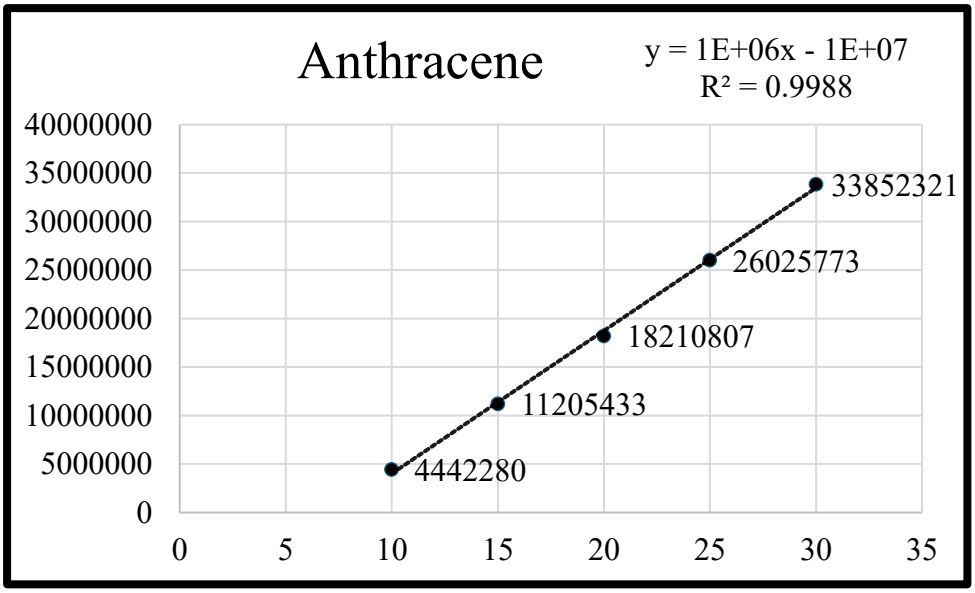


Figure: 5.14 Linearity curve of Anthracene

Table: 5.7 Results of Linearity for Anthracene

Linearity	Slope	Intercept	R^2
L-1	1E+06	1E+07	0.99
L-2	1E+06	1E+07	0.99
L-3	1E+06	1E+07	0.99
Mean	1E+06	1E+07	0.99

Table: 5.8 Back Calculated Values of Anthracene

Actual Concentration (µg/mL)	**10**	**15**	**20**	**25**	**30**
Obtained Concentration (µg/mL)	14.44	21.20	28.21	36.2	43.85
	14.38	21.38	28.19	36.14	43.97
	14.51	21.18	28.49	35.97	43.79
Avg.	14.44	21.26	28.26	36.04	43.87
SD	0.06	0.11	0.11	0.08	0.09
%CV	0.43	0.52	0.39	0.23	0.20

D) Linearity of Fluoranthene

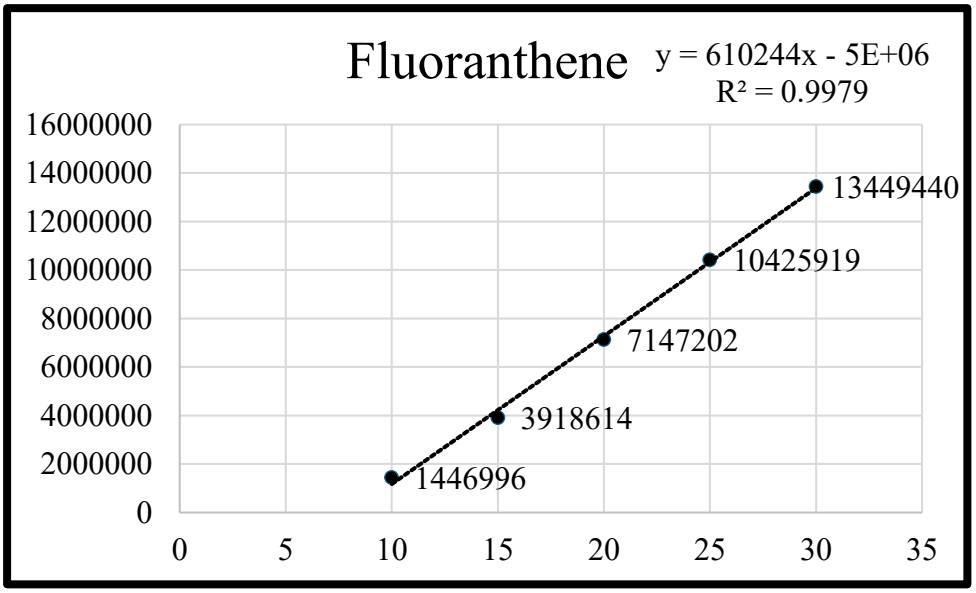


Figure: 5.15 Linearity curve of Fluoranthene

Table: 5.9 Results of Linearity for Fluoranthene

Linearity	Slope	Intercept	R^2
L-1	610244	5E+06	0.99
L-2	606324	5E+06	0.99
L-3	603112	5E+06	0.99
Mean	606560	5E+06	0.99

Table: 5.10 Back Calculated Values of Fluoranthene

Actual Concentration (µg/mL)	**10**	**15**	**20**	**25**	**30**
Obtained Concentration (µg/mL)	10.56	14.61	19.90	25.27	30.23
	10.88	14.67	19.89	25.37	30.53
	10.60	15.06	19.85	25.34	30.46
Avg.	10.68	14.78	19.88	25.33	30.41
SD	0.17	0.24	0.02	0.05	0.15
%CV	1.61	1.66	0.13	0.20	0.51

E) Linearity of Pyrene

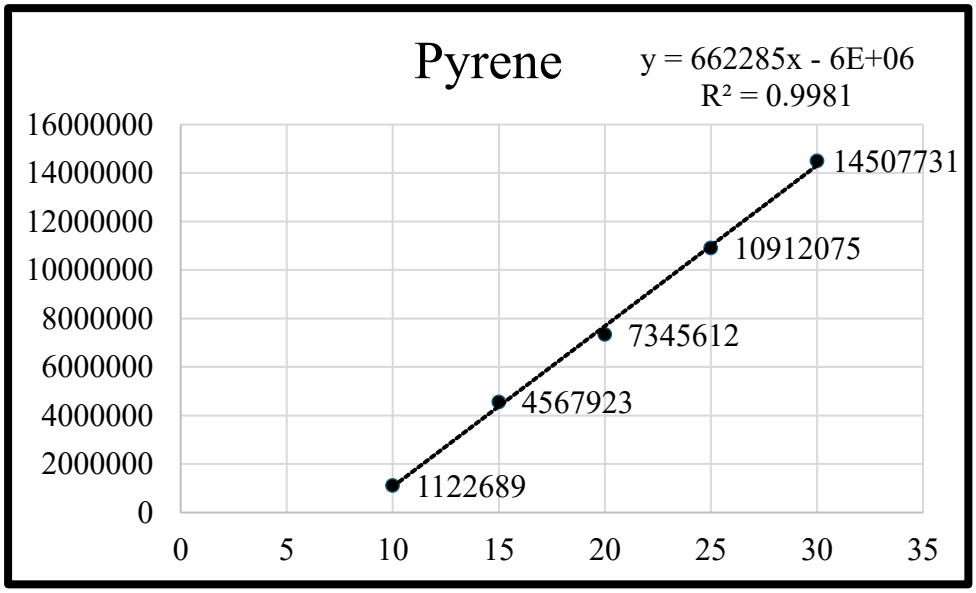


Figure: 5.16 Linearity curve of Pyrene

Table: 5.11 Results of Linearity for Pyrene

Linearity	Slope	Intercept	R^2
L-1	662285	6E+06	0.99
L-2	665284	6E+06	0.99
L-3	663907	5E+06	0.99
Mean	663825	5.66E+06	0.99

Table: 5.12 Back Calculated Values of Pyrene

Actual Concentration (µg/mL)	**10**	**15**	**20**	**25**	**30**
Obtained Concentration (µg/mL)	10.75	15.95	20.15	25.53	30.96
	10.65	15.65	19.97	25.33	30.81
	9.46	14.60	18.81	24.22	29.65
Avg.	10.29	15.40	19.64	25.03	30.48
SD	0.71	0.70	0.72	0.70	0.71
%CV	6.94	4.59	3.69	2.80	2.34

F) Linearity of Alpha-methyl Styrene

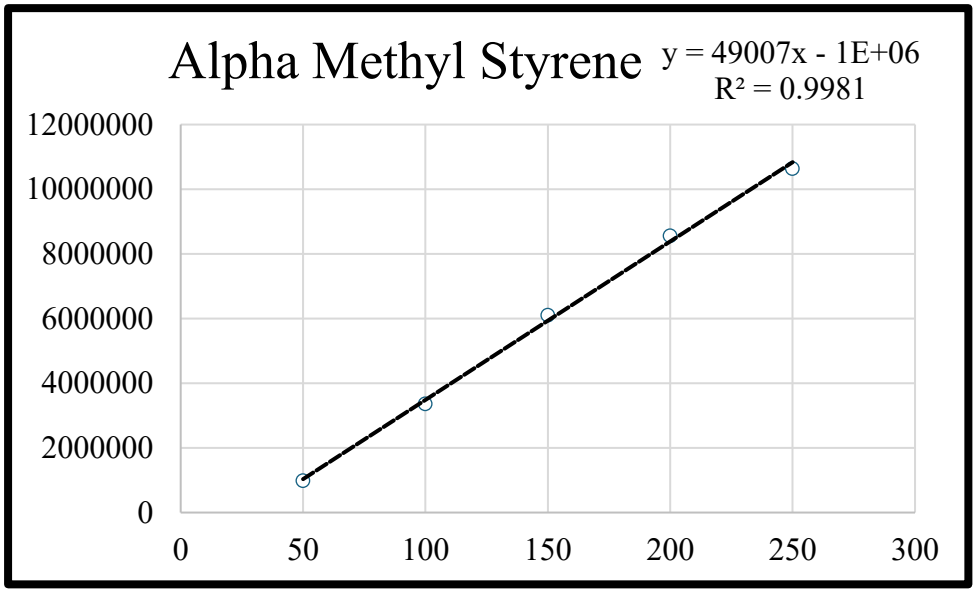


Figure: 5.17 Linearity curve of Alpha-methyl Styrene

Table: 5.13 Results of Linearity for Alpha-methyl Styrene

Linearity	Slope	Intercept	R^2
L-1	49007	1E+06	0.99
L-2	48279	1E+06	0.99
L-3	48778	1E+06	0.99
Mean	48688	1E+06	0.99

Table: 5.14 Back Calculated Values of Alpha Methyl Styrene

Actual Concentration (ng/mL)	**50**	**100**	**150**	**200**	**250**
Obtained Concentration (ng/mL)	40.65	89.06	145.00	195.22	237.58
	41.06	92.95	147.01	195.05	240.01
	40.97	87.90	143.22	198.83	235.50
Avg.	40.90	89.97	145.09	196.37	237.70
SD	0.21	2.64	1.87	2.13	2.25
%CV	0.52	2.93	1.29	1.08	0.94

G) Linearity of Diethyl Azelate

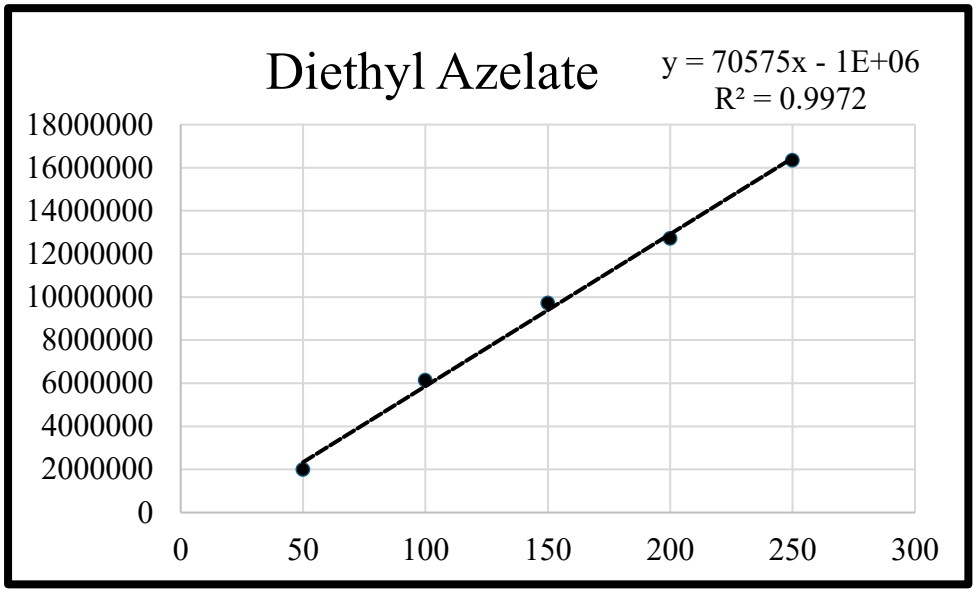


Figure: 5.18 Linearity curve of Diethyl Azelate

Table: 5.15 Results of Linearity for Diethyl Azelate

Linearity	Slope	Intercept	R^2
L-1	70575	1E+06	0.99
L-2	70760	1E+06	0.99
L-3	70688	1E+06	0.99
Mean	70674	1E+06	0.99

Table: 5.16 Back Calculated Values of Diethyl Azelate

Actual Concentration (ng/mL)	**50**	**100**	**150**	**200**	**250**
Obtained Concentration (ng/mL)	42.48	101.28	152.06	194.54	245.86
	41.00	100.29	151.05	193.68	244.62
	43.83	103.25	153.53	196.14	247.39
Avg.	42.44	101.61	152.21	194.79	245.95
SD	1.41	1.50	1.24	1.24	1.38
%CV	3.34	1.48	0.81	0.64	0.56

H) Linearity of Tributyrin

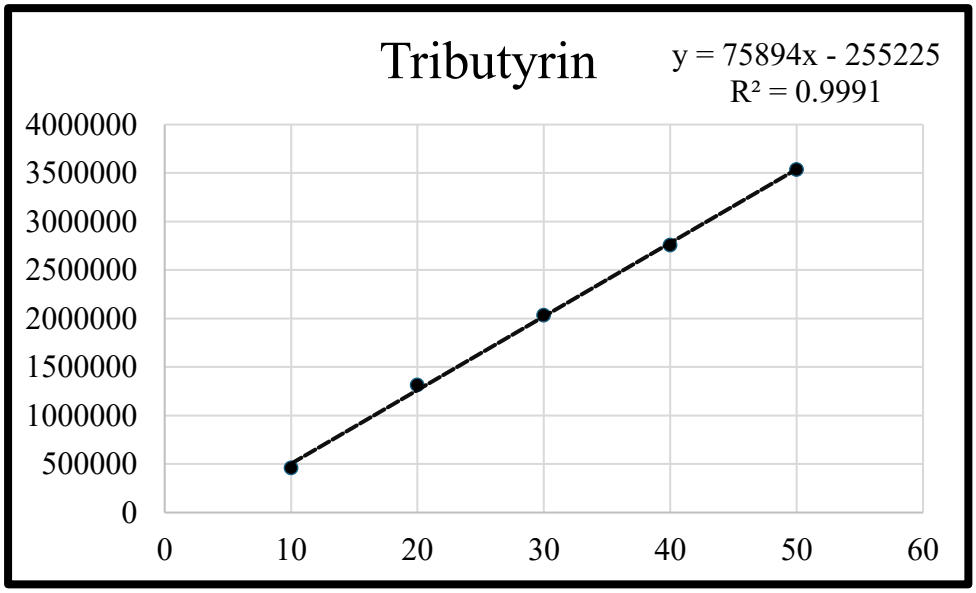


Figure: 5.19 Linearity curve of Tributyrin

Table: 5.17 Results of Linearity for Tributyrin

Linearity	Slope	Intercept	R^2
L-1	75894	255225	0.99
L-2	75780	259223	0.99
L-3	75809	238819	0.99
Mean	75827	251089	0.99

Table: 5.18 Back Calculated Values of Tributyrin

Actual Concentration (ng/mL)	**10**	**20**	**30**	**40**	**50**
Obtained Concentration (ng/mL)	9.45	20.70	30.18	39.69	49.96
	9.47	20.67	30.20	39.65	49.98
	9.48	20.67	30.17	39.68	49.97
Avg.	9.47	20.67	30.19	39.67	49.97
SD	0.01	0.01	0.01	0.02	0.01
%CV	0.12	0.06	0.05	0.05	0.02

- The range of an analytical technique is the range between the sample's higher and lower analyte concentrations (amounts), including these values, for which it has been shown that the analytical method has an adequate degree of linearity, precision, and accuracy.
- According to ICH Q2(R1) guideline at least five to six logs to the base-10 concentration levels should be covered within the linear working range. It is necessary to compute correlation coefficients or standard deviations across the whole linear dynamic range. Here for all eight analytes five concentration level linear working range was established for the validation. Also, the value of regression i.e. r^2 was found within the limit which is ≥0.990.

5.3.2 Accuracy

- Recovery of the standards were determined by mean of three times repetition of three concentration level (80%, 100% and 120%) in each standard.
- For the preparation of recovery samples, select a concentration from the decided range and considered that at 100% and make solution of 80 and 120% of that.
- Acceptance criteria of % recovery of each level should be within 98-102% i.e.±2% of 100%.

Table: 5.19 Results of Accuracy in Naphthalene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	20	16	36	7533132	35.3	98.13	
80	20	16	36	7917475	35.8	99.52	**98.70**
	20	16	36	7995960	35.4	98.38	

	20	20	40	10593031	39.4	98.42	
100	20	20	40	10692627	39.4	98.60	**99.20**
	20	20	40	11336667	40.3	100.70	
	20	24	44	13545051	43.2	98.09	
120	20	24	44	14355455	44.2	100.47	**99.30**
	20	24	44	14019495	43.8	99.47	

Table: 5.20 Results of Accuracy in Acenaphthene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	20	16	36	1221050	36.35	100.97	
80	20	16	36	1254329	36.71	101.97	**100.90**
	20	16	36	1186669	35.97	99.917	
	20	20	40	1621050	40.68	101.7	
100	20	20	40	1554329	39.95	99.87	**100.70**
	20	20	40	1586669	40.3	100.75	
	20	24	44	1843130	43.08	97.90	
120	20	24	44	1973937	44.5	101.14	**100.40**
	20	24	44	2019281	44.99	102.25	

Table: 5.21 Results of Accuracy in Anthracene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	20	16	36	57019392	36.35	100.97	
80	20	16	36	59563443	36.71	101.97	**99.70**
	20	16	36	63497433	35.97	99.91	
	20	20	40	74456665	40.68	101.7	
100	20	20	40	77304150	39.95	99.87	**99.40**
	20	20	40	77155089	40.3	100.75	
	20	24	44	96259625	43.08	97.90	
120	20	24	44	93610716	44.5	101.14	**99.40**
	20	24	44	97615938	44.99	102.25	

Table: 5.22 Results of Accuracy in Fluoranthene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	20	16	36	68277644	36.28	100.78	
80	20	16	36	67268409	35.9	99.72	**100.00**
	20	16	36	67267969	35.88	99.66	
	20	20	40	88358520	39.6	99	
100	20	20	40	89358793	39.61	99.02	**99.30**
	20	20	40	87367113	39.95	99.87	

	20	24	44	102467103	44.05	100.11	
120	20	24	44	100451231	43.5	98.86	**99.40**
	20	24	44	100457999	43.68	99.27	

Table: 5.23 Results of Accuracy in Pyrene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	20	16	36	8531040	35.4	98.33	
80	20	16	36	8767339	35.64	99	**99.50**
	20	16	36	8886768	36.42	101.17	
	20	20	40	9622050	40.27	100.68	
100	20	20	40	9555329	40.34	100.85	**99.80**
	20	20	40	9586769	39.18	97.95	
	20	24	44	1053230	43.46	98.77	
120	20	24	44	1063837	43.28	98.36	**99.50**
	20	24	44	1079271	44.69	101.57	

Table: 5.24 Results of Accuracy in Alpha- methyl Styrene

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	15	12	27	6883097	26.75	99.0741	
80	15	12	27	6955134	27.03	100.111	**98.10**
	15	12	27	6625785	25.75	95.3704	

	15	15	30	8774800	30.35	101.167	
100	15	15	30	9003175	31.14	103.8	**100.10**
	15	15	30	8274594	28.62	95.4	
	15	18	33	12641562	32.65	98.9394	
120	15	18	33	12653176	32.68	99.0303	**98.20**
	15	18	33	12382149	31.98	96.9091	

Table: 5.25 Results of Accuracy in Diethyl Azelate

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	15	12	27	20900995	27.96	103.556	
80	15	12	27	21125231	28.26	104.667	**101.60**
	15	12	27	19525444	26.12	96.7407	
	15	15	30	79065195	29.32	97.7333	
100	15	15	30	81762122	30.32	101.067	**98.60**
	15	15	30	78525922	29.12	97.0667	
	15	18	33	51010876	33.65	101.97	
120	15	18	33	51389568	33.9	102.727	**100.80**
	15	18	33	49024868	32.34	98	

Table: 5.26 Results of Accuracy in Tributyrin

Level (%)	Concentration volume added (µg/mL) Std	Concentration volume taken (µg/mL) Test	Concentration volume found (µg/mL) Total	Area	Obtained concentration (µg/mL)	% Recovery	Average % Recovery
	3.0	2.4	5.4	417654	5.34	98.8889	

80	3.0	2.4	5.4	435642	5.57	103.148	**101.20**
	3.0	2.4	5.4	429306	5.48	101.648	
	3.0	3.0	6.0	1705145	6.10	101.667	
100	3.0	3.0	6.0	1646438	5.89	98.1667	**100.80**
	3.0	3.0	6.0	1721916	6.16	102.667	
	3.0	3.6	6.6	3447381	6.54	99.0909	
120	3.0	3.6	6.6	3421024	6.49	98.3333	**100.20**
	3.0	3.6	6.6	3594964	6.82	103.333	

- A minimum of nine determinations over a minimum of three concentration levels within the designated range should be used to evaluate accuracy (e.g., 3 concentrations/3 replicates each of the entire analytical technique). Accuracy should be expressed as the difference between the mean and the recognised true value, together with the confidence intervals, or as the percentage recovery by the assay of the known additional amount of analyte in the sample.
- According to ICH Q2(R1) guideline 3 concentration level in 3 replicates total 9 sample analysis should be done to establish the accuracy with the percentage recovery metrics. In the present work accuracy for all eight analytes were performed and the value of percentage recovery metrics was near by 100 percent i.e. 100±2 percent value.

5.3.3 Precision

- Precision was performed by using six replicates of any one concentration from linearity range.
- Precision was calculated by measuring standard deviation and %CV of each concentration levels.

- Precision was performed by two different methods, i.e. intraday precision and interday precision.
- The precision, which was performed two times a day, one at morning and another one at evening by using six replicated of decided concentration known as intraday precision.
- Interday precision was performed at different days that is mentioned as day 1, day 2, day 3 by using six replicates of decided concentration.

Table: 5.27 Results of Precision in Naphthalene

	Intraday		Interday	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	7236914	6236924	6336934	6080313
	6641487	7641477	7741487	6336935
	7045190	7045180	7145190	6045180
	6883905	7883995	7983985	5883815
	6450717	7450707	7550717	6044180
	6197225	7197215	7297225	6741586
Average	6742573	7242583	7342590	6188668
SD	386969.70	577272.50	577268.0	307845.10
%CV	5.73	7.97	7.86	4.97

Table: 5.28 Results of Precision in Acenaphthene

	Intraday		Interday	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	7286100	6287110	7286100	6277210
	7954472	6955482	7954472	6945582

	7384447	6385457	7384447	6375557
	7593883	6594893	7593883	6584993
	7584131	6585141	7584131	6575241
	6930952	6931962	6930952	6921862
Average	7455664	6623341	7455664	6613408
SD	344547.30	274762.90	344547.30	274718.00
%CV	4.62	4.14	4.62	4.15

Table: 5.29 Results of Precision in Anthracene

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	18714909	28724909	28724909	18210807
	17954571	27955571	27955571	20724919
	19372373	29373373	29373373	19472383
	20398029	30399029	30399029	19398135
	19938025	29939025	29939025	20373475
	18950508	28951508	28951508	17955432
Average	19221403	29223903	29223903	19355859
SD	878131.1	877100.0	877100.0	1113335.0
%CV	4.56	3.00	3.00	5.75

Table: 5.30 Results of Precision in Fluoranthene

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	8505278	9506279	9006284	7147202
	7725828	8726829	9226834	7006274

	7665233	8666234	9166239	7265239
	8848051	9849052	10349057	6848555
	7595564	8596565	9096570	6666147
	8188379	9189380	9689385	7226952
Average	8088056	9089057	9422395	7026728
SD	512692.50	512692.50	512475.60	233954.60
%CV	6.33	5.64	5.43	3.32

Table: 5.31 Results of Precision in Pyrene

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	9727287	9626277	10626277	7345612
	9868071	9767061	10767061	7626287
	8968272	8867262	9867262	7968282
	10273124	9272114	10272114	8273420
	8589884	8488874	9488874	7867565
	8663354	8562344	9562344	8767684
Average	9348332	9097322	10097322	7974808
SD	701121.90	541891.90	541891.90	499343.50
%CV	7.49	5.95	5.36	6.26

Table: 5.32 Results of Precision in Alpha-methyl Styrene

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	4963646	4740478	5098712	5183942
	4542218	4830926	4798712	5281939

	4620835	4901287	4809856	4928239
	4985934	4999631	5198765	4629482
	4869269	4816532	4698479	4720402
	5251550	4710278	4787642	4624729
Average	4872242	4833189	4898694	4894789
SD	259725.00	106137.00	200178.40	285777.70
%CV	5.33	2.19	4.08	5.83

Table: 5.33 Results of Precision in Diethyl Azelate

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	4865892	5309765	5294631	5082839
	4759891	4609261	4698510	4820384
	4891172	4891287	5198364	5129302
	5071650	5298711	4792846	4928232
	4970757	4798123	5082646	5018273
	5248414	5012874	4808362	4898721
Average	4967963	4986670	4979227	4979625
SD	172638.90	279117.20	245328.70	117553.70
%CV	3.47	5.59	4.92	2.36

Table: 5.34 Results of Precision in Tributyrin

	Intraday		**Interday**	
	Precision 1 (Morning)/ Day 1	**Precision 2 (Evening)**	**Day 2**	**Day 3**
Peak Area	37112040	3701287	3917384	3982739
	37445842	4209864	3891836	4029284

	37704140	3987612	3917384	3681839
	36090663	3898762	3793490	3619302
	40301713	3801298	4028474	3482744
	39843306	3787611	3810384	4029382
Average	38082951	3897739	3893159	3804215
SD	1642135.00	181946.50	85171.84	239056.20
%CV	4.31	4.66	2.18	6.28

- The variance, standard deviation, or coefficient of variation of a set of measurements is typically used to represent the precision of an analytical process. Repeatability is the ability to convey precision over a brief period under the same operational conditions. Another name for repeatability is intra-assay precision. Variations within laboratories, such as various days, different analysers, different equipment, etc., are expressed by intermediate precision. Both the methods of precision were established using six replicates of each time at a same concentration level.
- At this point in this work the computation of precision was done by establishing the value of percentage coefficients of variation %CV was found within the acceptable range i.e. less than 20 percent. For all eight analytes value of the precision was within the acceptance measures as per ICH Q2(R1) guideline.

5.3.4 Robustness

- Robustness of method was performed for one concentration and analysed by standard deviation and %RSD.
- To perform robustness flow rate, temperature and hold time and pressure was changed from set parameters to ±2, three replicates were used for each level.

The acceptance criteria %RSD of each level was ≤2% for lower-level impurities the acceptance criteria of %RSD can be 5- 10 %.

5.3.4.1 Robustness for Change in Flow Rate

- The change in flow rate of the developed method was made from the actual flow rate. For the group one of the developed methods actual flow rate was 15.5 mL/min and 24.3 mL/min for group two was set. To the actual value ±2 mL/min change in flow rate was measured in three replicates.

Table: 5.35 Results of Robustness (flow rate) in Naphthalene

	13.5 mL/min	15.5 mL/min	17.5 mL/min
Peak Area	6987996	4580313	5251142
	6789766	4495205	5364729
	6864559	4589135	5096852
Average	6880773	4554884	5237574
SD	100104.79	51871.70	134453.00
%RSD	**1.45**	**1.13**	**2.56**

Table: 5.36 Results of Robustness (flow rate) in Acenaphthene

	13.5 mL/min	15.5 mL/min	17.5 mL/min
Peak Area	9343431	5699321	7584209
	9497420	5930294	7940294
	9305629	5830593	7602491
Average	9382160	5820069	7708998
SD	101591.80	115845.60	200517.00
%RSD	**1.08**	**1.99**	**2.60**

Table: 5.37 Results of Robustness (flow rate) in Anthracene

	13.5 mL/min	15.5 mL/min	17.5 mL/min
Peak Area	24540269	14210807	18891385
	28401573	15093764	20395347
	27405612	14096735	21856493
Average	26782485	14467102	20381075
SD	2004653.00	545694.10	1482606.00
%RSD	**7.48**	**3.77**	**7.27**

Table: 5.38 Results of Robustness (flow rate) in Fluoranthene

Peak Area	**13.5 mL/min**	**15.5 mL/min**	**17.5 mL/min**
	9744496	6147202	7530217
	9405827	6738494	7749284
	9674628	6836452	7946463
Average	9608317	6574049	7741988
SD	178807.20	372891.00	208219.00
%RSD	**1.86**	**5.67**	**2.68**

Table: 5.39 Results of Robustness (flow rate) in Pyrene

	13.5 mL/min	15.5 mL/min	17.5 mL/min
Peak Area	9850112	7145612	7713822
	9923840	7493847	7840593
	10293843	7394847	8049573
Average	10022598	7344769	7867996
SD	237779.70	179437.40	169545.00
%RSD	**2.37**	**2.44**	**2.15**

Table: 5.40 Results of Robustness (flow rate) in Alpha-methyl Styrene

	22.3 mL/min	24.3 mL/min	26.3 mL/min
Peak Area	8631548	6106433	7238390
	8694612	6016944	7395467
	8408277	6072973	7147290
Average	8578145.66	6065450.00	7260382.33
SD	150451.95	45216.33	125541.63
%RSD	**1.75**	**0.74**	**1.72**

Table: 5.41 Results of Robustness (flow rate) in Diethyl Azelate

	22.3 mL/min	24.3 mL/min	26.3 mL/min
Peak Area	12146550	9731876	10884274
	12210840	9712749	10930190
	12153738	9844588	10793066
Average	12170376	9763071	10869176.7
SD	35226.67	71240.62	69797.52
%RSD	**0.28**	**0.72**	**0.64**

Table: 5.42 Results of Robustness (flow rate) in Tributyrin

	22.3 mL/min	24.3 mL/min	26.3 mL/min
Peak Area	4539534	2035737	3148264
	4466970	1971937	3196280
	4496541	2046518	3053781
Average	4501015	2018064	3132775
SD	36488.29	40309.21	72501.19
%RSD	**0.81**	**1.99**	**2.31**

5.3.4.2 Robustness for Change in Temperature and Hold Time

- The change in temperature and hold time of the developed method was made from the actual temperature and hold time. For the group one of the developed methods actual temperature and hold time was optimized 250˚c (20min) and 200˚c (10min) for group two was set. To the actual value ±2 ˚c and time in min change in temperature and hold time was measured in three replicates.

Table: 5.43 Results of Robustness (temperature and hold time) in Naphthalene

	248˚c (18min)	250˚c (20min)	252˚c (22min)
Peak Area	5922633	4580313	7326364
	5812934	4495205	7134789
	6085346	4589135	7435689
Average	5940304	4554884	7298947
SD	137063.00	51871.70	152312.00
%RSD	**2.30**	**1.13**	**2.08**

Table:5.44 Results of Robustness (temperature and hold time) in Acenaphthene

	248˚c (18min)	250˚c (20min)	252˚c (22min)
Peak Area	8180237	5699321	8639189
	8264738	5930294	8862553
	8382739	5830593	8725272
Average	8275905	5820069	8742338
SD	101711.80	115845.60	112656.00
%RSD	**1.22**	**1.99**	**1.28**

Table: 5.45 Results of Robustness (temperature and hold time) in Anthracene

	248˚c (18min)	250˚c (20min)	252˚c (22min)
Peak Area	20203432	14210807	19332008
	22846349	15093764	18254552

	21846469	14096735	20745277
Average	21632083	14467102	19443946
SD	1334438.00	545694.10	1249130.00
%RSD	**6.16**	**3.77**	**6.42**

Table: 5.46 Results of Robustness (temperature and hold time) in Fluoranthene

	248˚c (18min)	**250˚c (20min)**	**252˚c (22min)**
Peak Area	7717282	6147202	8642721
	7682929	6738494	8725544
	7826466	6836452	8862544
Average	7742225	6574049	8743603
SD	74949.00	372891.00	111019.00
%RSD	**0.96**	**5.67**	**1.26**

Table: 5.47 Results of Robustness (temperature and hold time) in Pyrene

	248˚c (18min)	**250˚c (20min)**	**252˚c (22min)**
Peak Area	9691025	7145612	9113491
	9736253	7493847	9274889
	9825466	7394847	9375839
Average	9750915	7344769	9254740
SD	68409.2	179437.4	132330.0
%RSD	**0.70**	**2.44**	**1.42**

Table: 5.48 Results of Robustness (temperature and hold time) in Alpha-methyl Styrene

	198˚c (08min)	**200˚c (10min)**	**202˚c (12min)**
Peak Area	7947629	6106433	9143782
	7821574	6016944	9032491
	7953158	6072973	9094483
Average	7907453.667	6065450	9090252

SD	74425.33	45216.33	55766.00
%RSD	**0.94**	**0.74**	**0.61**

Table: 5.49 Results of Robustness (temperature and hold time) in Diethyl Azelate

	198˚c (08min)	**200˚c (10min)**	**202˚c (12min)**
Peak Area	7643860	9731876	8056072
	7694412	9712749	8240376
	7743185	9844588	8139541
Average	7693819	9763071	8145329.67
SD	49665.15	71240.62	92288.25
%RSD	**0.64**	**0.72**	**1.13**

Table: 5.50 Results of Robustness (temperature and hold time) in Tributyrin

	198˚c (08min)	**200˚c (10min)**	**202˚c (12min)**
Peak Area	2953479	2035737	3578425
	2849366	1971937	3496581
	2874346	2046518	3541586
Average	2892397	2018064	3538864
SD	54353.08	40309.21	40989.84
%RSD	**1.87**	**1.99**	**1.15**

5.3.4.3 Robustness for Change in Pressure

- The change in pressure of the developed method was made from the actual pressure. For the group one of the developed methods actual pressure was optimized 134.3 kPa and 103.5 kPa for group two was set. To the actual value ±2 kPa change in pressure was measured in three replicates.

Table: 5.51 Results of Robustness (Pressure) in Naphthalene

	132.3 kPa	134.3 kPa	136.3 kPa
Peak Area	6422628	4580313	5634766
	6586848	4495205	5589670
	6635960	4589135	5660600
Average	6548478	4554884	5628345
SD	111721.93	51871.70	35898.30
%RSD	**1.70**	**1.13**	**0.63**

Table: 5.52 Results of Robustness (Pressure) in Acenaphthene

	132.3 kPa	134.3 kPa	136.3 kPa
Peak Area	7665148	5699321	7410630
	7735744	5930294	7524889
	7634567	5830593	7653346
Average	7678486	5820069	7529622
SD	51890.55	115845.60	121427.00
%RSD	**0.67**	**1.99**	**1.61**

Table: 5.53 Results of Robustness (Pressure) in Anthracene

	132.3 kPa	134.3 kPa	136.3 kPa
Peak Area	18246221	14210807	18440777
	19743890	15093764	17579060
	18654435	14096735	17643479
Average	18881515	14467102	17887772
SD	774226.90	545694.10	479998.30
%RSD	**4.10**	**3.77**	**2.68**

Table: 5.54 Results of Robustness (Pressure) in Fluoranthene

	132.3 kPa	134.3 kPa	136.3 kPa
Peak Area	6808954	6147202	6390264
	6759589	6738494	6595848
	6648495	6836452	6596957
Average	6739012	6574049	6527690
SD	82184.62	372891.00	119015.00
%RSD	**1.21**	**5.67**	**1.82**

Table: 5.55 Results of Robustness (Pressure) in Pyrene

	132.3 kPa	134.3 kPa	136.3 kPa
Peak Area	8262459	7145612	7314552
	8147586	7493847	7444859
	8337596	7394847	7396969
Average	8249214	7344769	7385460
SD	95694.98	179437.40	65911.50
%RSD	**1.16**	**2.44**	**0.89**

Table: 5.56 Results of Robustness (Pressure) in Alpha-methyl Styrene

	101.5 kPa	103.5 kPa	105.5 kPa
Peak Area	8175837	6106433	7528439
	8046732	6016944	7614575
	8166574	6072973	7487990
Average	8129714.333	6065450	7543668
SD	72013.89	45216.33	64652.00
%RSD	**0.88**	**0.74**	**0.85**

Table: 5.57 Results of Robustness (Pressure) in Diethyl Azelate

	101.5 kPa	103.5 kPa	105.5 kPa
Peak Area	7952228	9731876	7148293
	7836725	9712749	7085691
	7890433	9844588	7157638
Average	7893128.67	9763071	7130540.67
SD	57798.66	71240.62	39120.98
%RSD	**0.73**	**0.72**	**0.54**

Table: 5.58 Results of Robustness (Pressure) in Tributyrin

	101.5 kPa	103.5 kPa	105.5 kPa
Peak Area	3936633	2035737	4388795
	3990483	1971937	4256371
	4027947	2046518	4270348
Average	3985021	2018064	4305171.33
SD	45901.38	40309.21	72756.63
%RSD	**1.15**	**1.99**	**1.68**

- Robustness testing ought to demonstrate an analytical procedure's dependability regarding intentional parameter changes. It is in place to guarantee that the analytical procedure's validity is always upheld. As per the above description the metrics of robustness computation was established using three parameters variation into three replicates of each. Measurements of the robustness was done by using %RSD measurement. The value of %RSD was found within the acceptance criteria as mentioned.

5.3.5 LOD and LOQ

- LODs were estimated by analysing standards at different concentration levels. The criteria used for determining these limits were the retention time window (RTW). The values of LOD are given in table 5.52.
- The LOQ is the lowest level of the validation meeting these method performance acceptability criteria.
- LOQs were determined according to the minimum concentration that may provide suitable recovery.

Table: 5.59 Results of LOD and LOQ

Name of Analytes	LOD	LOQ
Naphthalene	0.05	0.16
Acenaphthene	03.30	10.00
Anthracene	01.49	04.54
Fluoranthene	02.02	06.14
Pyrene	02.23	06.77
Alpha-methyl Styrene	01.90	05.77
Diethyl Azelate	01.11	03.38
Tributyrin	01.10	03.36

- As per the guideline ICH Q2(R1) the value of detection limit and quantification limit is showing the signal-to-noise ratio in the ratio pf 10:1 and 3:1 respectively. The values of this ratio were calculated by using standard deviation and intercept of the linearity data. All the computed data falls within the criteria.

5.3.6 Specificity

- Specificity of the standards were determined by blank solution which contained only a diluent that is acetone in this case.

- Here, no peak observed at a retention time of the standards. Only a peak of diluent is observed in given chromatogram.

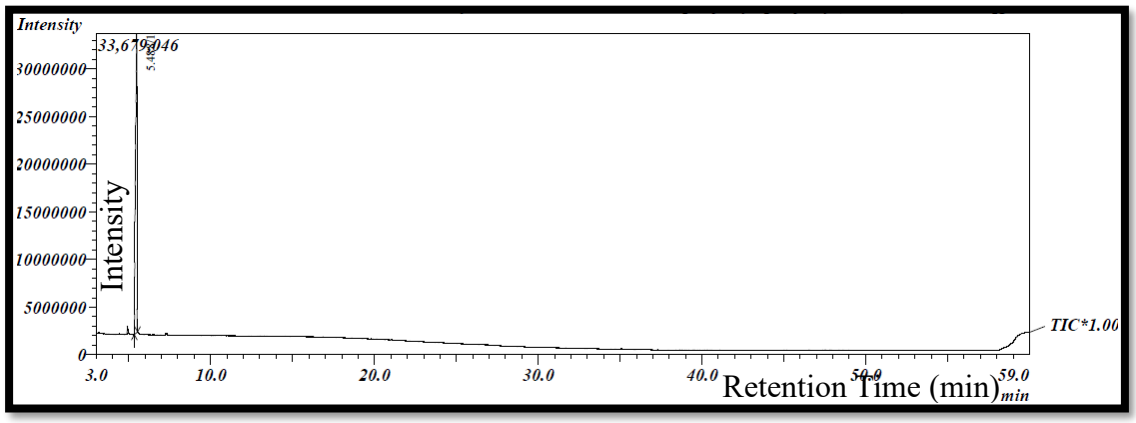


Figure: 5.20 Chromatogram of Specificity for Group 1 (naphthalene, acenaphthene, anthracene, fluoranthene and pyrene)

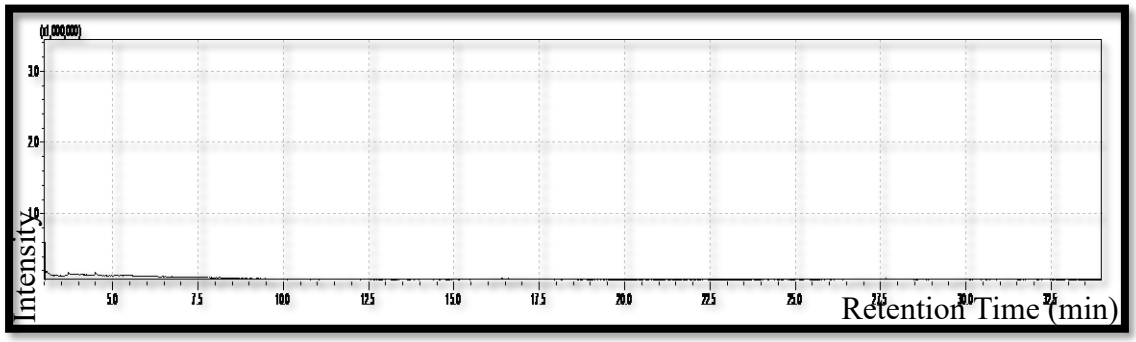


Figure: 5.21 Chromatogram of Specificity for Group 2 (alpha-methyl styrene, diethyl azelate and tributyrin)

- The capacity to definitively evaluate the analyte in the presence of potentially predicted components is known as specificity. To guarantee the identity of an analyte, these may typically contain contaminants, degradants, matrix, etc.

- As per figure number 5.20 and 5.21 the chromatogram of the black is showing no peak or no interference of other impurities. According to the guidelines ICH Q2(R1) the criteria is fulfilled by the developed method.

5.5 Application results of developed method

Table: 5.60 Optimized extraction techniques and conditions

Sr no.	Extraction Techniques	Solvent Used	Conditions Applied
1	Reflux Condensation	n-Hexane	6 Hrs. at 55˚c (Water Bath)
2	pH	Phosphate Buffer: 2.5pH Ammonia Buffer: 9.5pH	3-4 Days at 50-70˚c (Hot Air Oven)
3	Sunlight	-	About 2 Months

- Both the developed methods have been successfully applied on some pharmaceutical formulations and the chromatograms are given below.

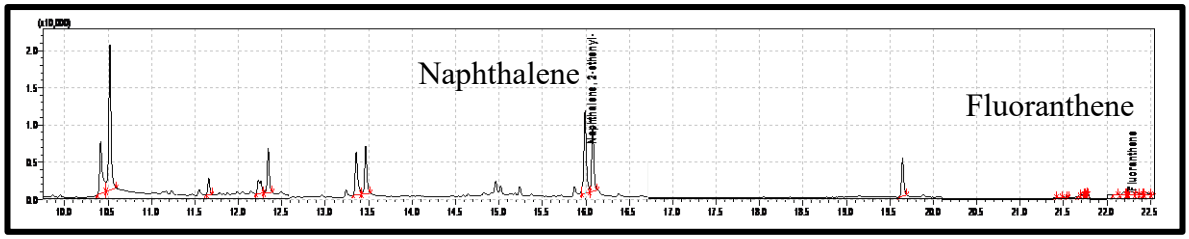


Figure: 5.22 Results of Reflux condensation from Eye Drops

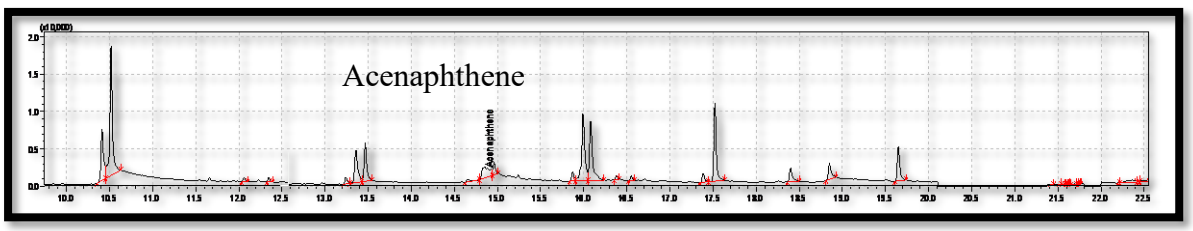


Figure: 5.23 Results of Reflux condensation from SWFI

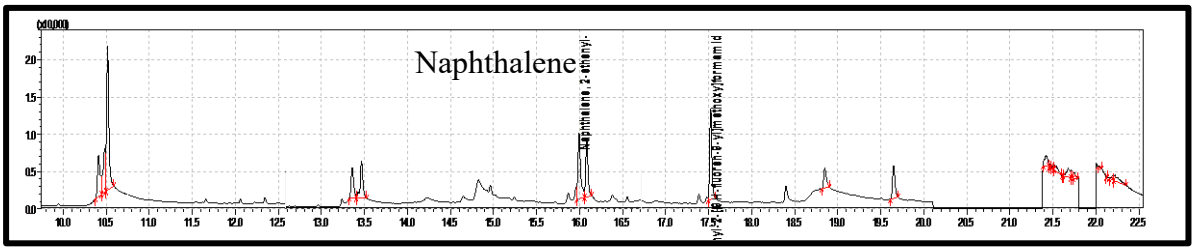


Figure: 5.24 Results of Reflux condensation from NS

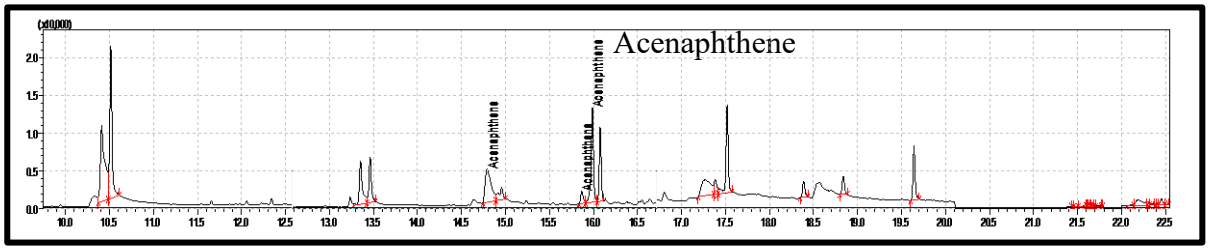


Figure: 5.25 Results of pH from Ear Drops

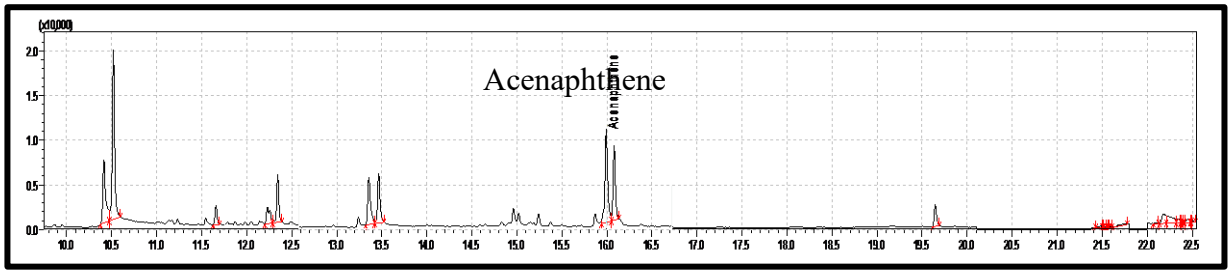


Figure: 5.26 Result of pH from Eye Drops

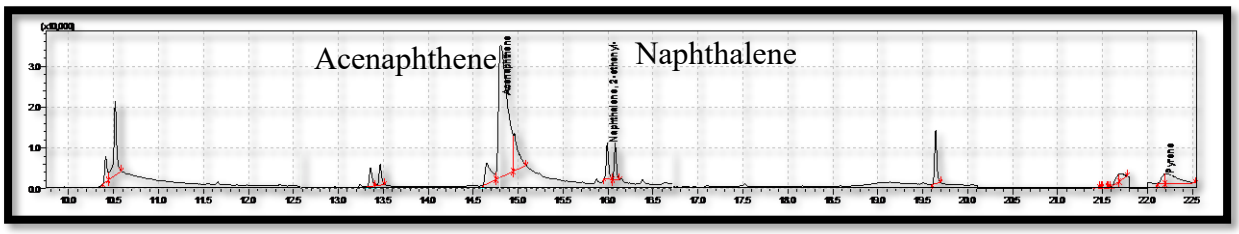


Figure: 5.27 Result of pH from SWFI

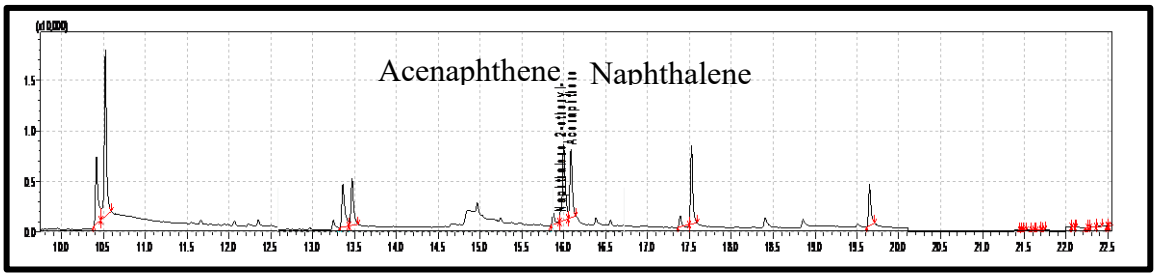


Figure: 5.28 Result of pH from NS

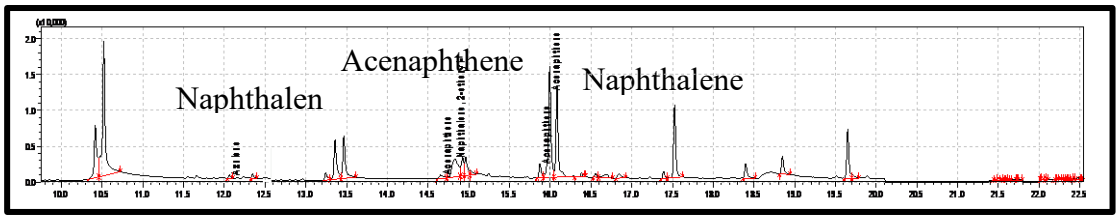


Figure: 5.29 Result of Sunlight from Eye Drops

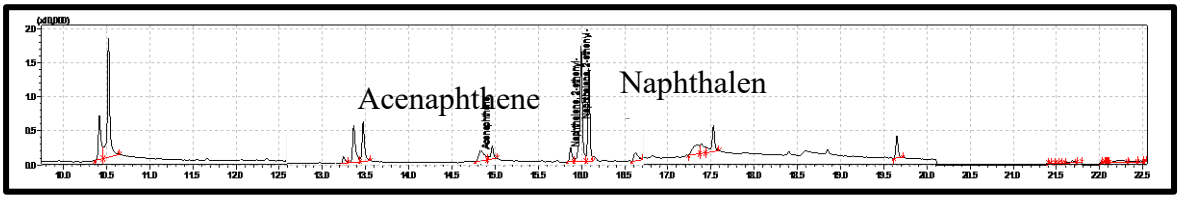


Figure: 5.30 Result of Sunlight from Ear Drops

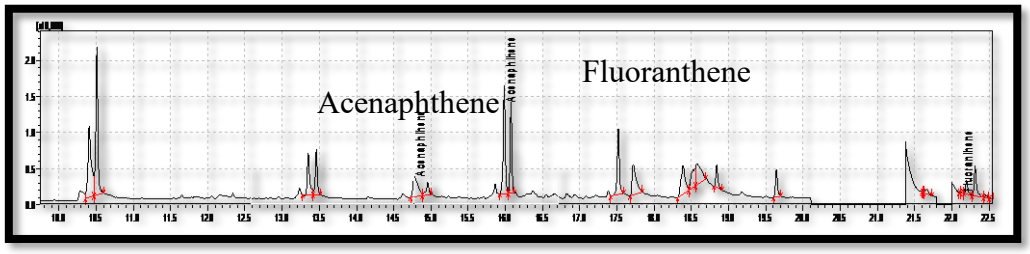


Figure: 5.31 Result of Sunlight from NS

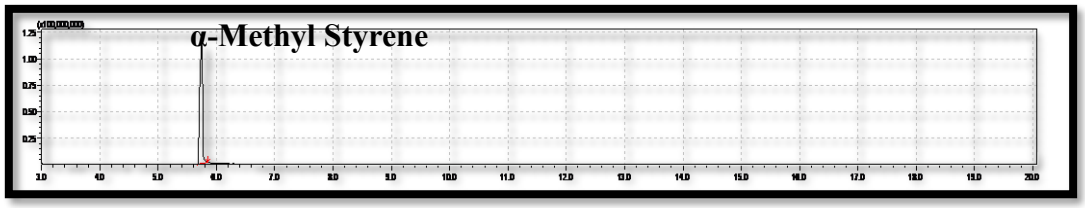


Figure: 5.32 Result of Reflux from Ciprofloxacin

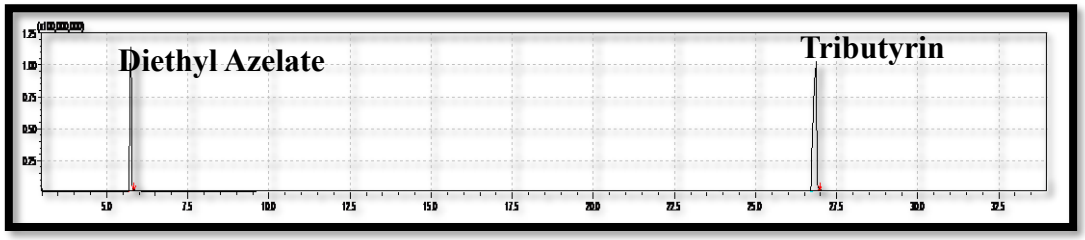


Figure: 5.33 Result of Reflux from 25D

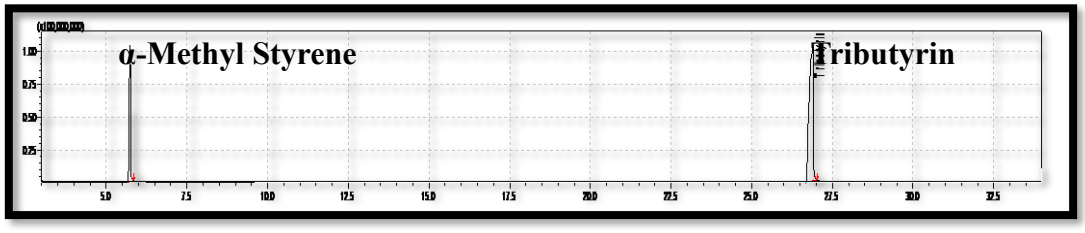


Figure: 5.34 Result of Reflux from Metronidazole

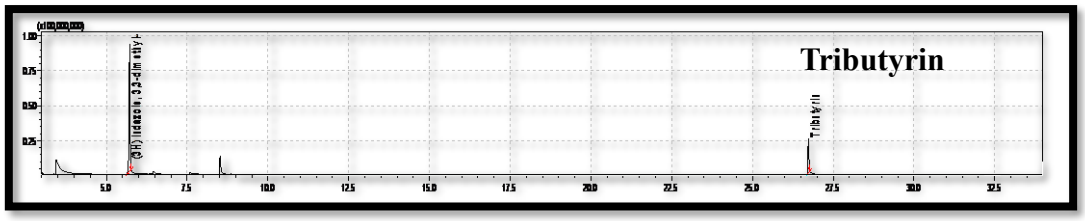


Figure: 5.35 Result of pH from Ciprofloxacin

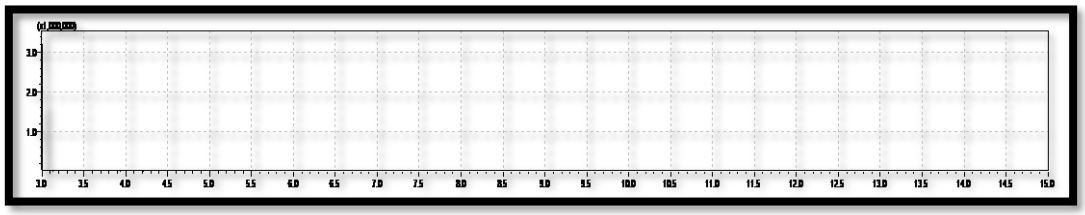


Figure: 5.36 Result of pH from 25D

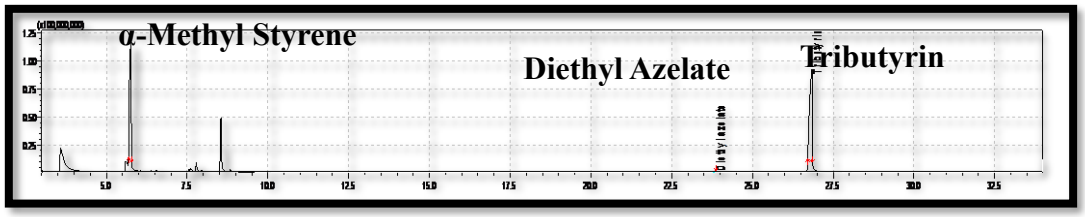


Figure: 5.37 Result of pH from Metronidazole

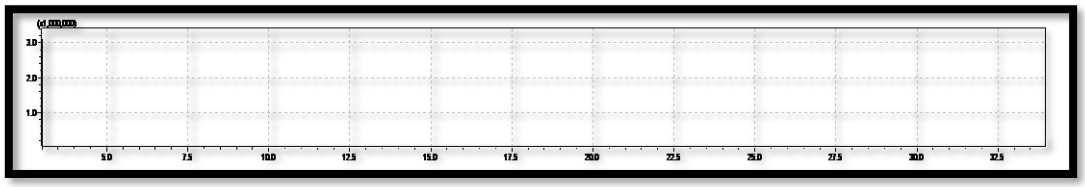


Figure: 5.38 Result of Sunlight from Ciprofloxacin

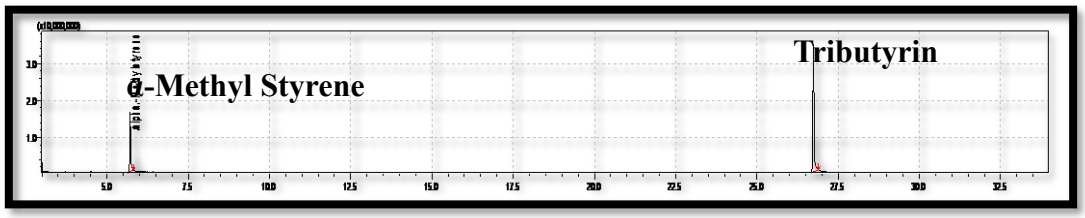


Figure: 5.39 Result of Sunlight from 25D

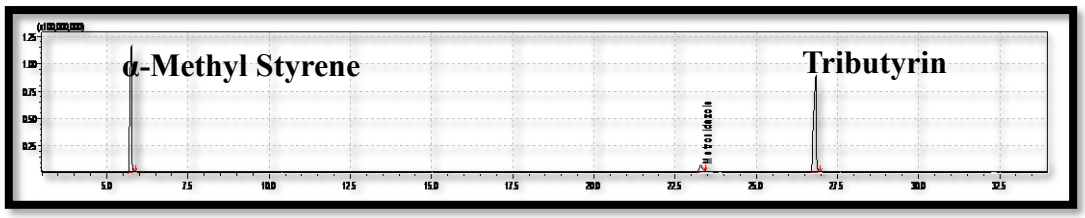


Figure: 5.40 Result of Sunlight from Metronidazole

- Developed method is validated and categorized for group 1 and group 2 for the identification of all the analytes with ease. Also, the separation of all the analytes and resolution of all the analytes was recognised properly.

Table: 5.61 Analytical data summary of group 1

Sr no.	Extraction Technique	Eye Drops (µg/mL)	Ear Drops (µg/mL)	SWFI (µg/mL)	NS (µg/mL)
1	**Reflux**	Naphthalene (0.32) Fluoranthene (0.61)	**Not Observed**	Acenaphthene (1.00)	Naphthalene (2.33)
2	**pH**	Acenaphthene (1.11)	Acenaphthene (1.11)	Acenaphthene (1.10) Naphthalene (4.68)	Acenaphthene (0.10) Naphthalene (17.79)
3	**Sunlight**	Acenaphthene (1.16) Naphthalene (6.18)	Acenaphthene 1.03) Naphthalene (9.60)	**Not Observed**	Acenaphthene (1.01) Fluoranthene (0.61)

Table: 5.62 Analytical data summary of group 2

Sr no.	Extraction Technique	Ciprofloxacin (µg/mL)	25D (µg/mL)	Metronidazole (µg/mL)
1	**Reflux**	α- Methyl Styrene (0.15)	Diethyl Azelate (0.10) Tributyrin (0.96)	α- Methyl Styrene (0.11) Tributyrin (1.17)
2	**pH**	Tributyrin (0.07)	**Not Observed**	α- Methyl Styrene (0.15) Diethyl Azelate (0.55) Tributyrin (0.58)
3	**Sunlight**	**Not Observed**	α- Methyl Styrene (0.24) Tributyrin (0.50)	α- Methyl Styrene (0.20) Tributyrin (0.66)

5.6 Toxicological Study

5.6.1 Optimized Cell Line Methodology for both groups

5.6.1.1 Preparation of Solution 1:

100mg of sample was taken and dissolved in 1mL of DMSO. Solution was undergone the vertex mixture until it gets completely soluble.

5.6.1.2 Preparation of Solution 2:

0.1mL sample was withdrawn from solution 1 and make up to 1mL with Water (Approx 0.9mL). after edition white coloured precipitates or hazy solution appeared on mixing. To overcome this problem solution were kept on dry hot bath (at 50˚c until complete dissolution) and marked as solution 2.

5.6.1.3 Sample Application:

100µL from solution 2 was taken and media was added. Afterwards well mixing was provided. The final solution or sample was introduced to the first well of well plate in a triplicate (1000µg/mL).

5.6.1.4 Serial Dilution:

100µL solution from the 1st well was taken and added to the 2nd well of well plate. The same process was repeated for up to 6 well.

5.6.1.5 Negative Control

On the well plate, 6th well has been kept with cell and media only (without Sample) which will appeared as a negative control of the study.

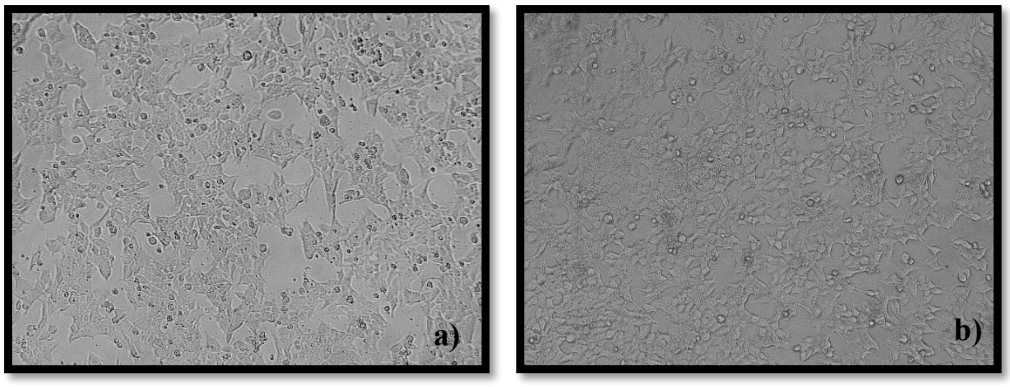


Figure: 5.41 Effect of Negative Control on Cell line a) HEK and b) MCF7

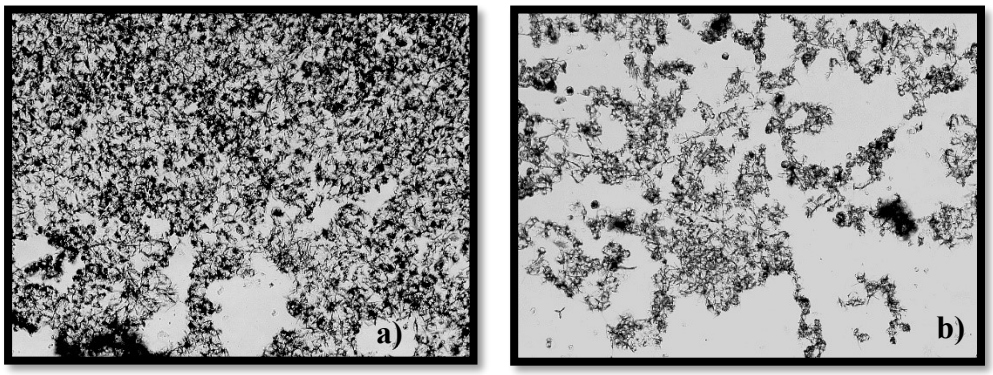


Figure: 5.42 Formation of Crystals in Cell line a) HEK and b) MCF7

5.6.2 Effect of Different Concentration of Analytes on the Selected Cell Lines

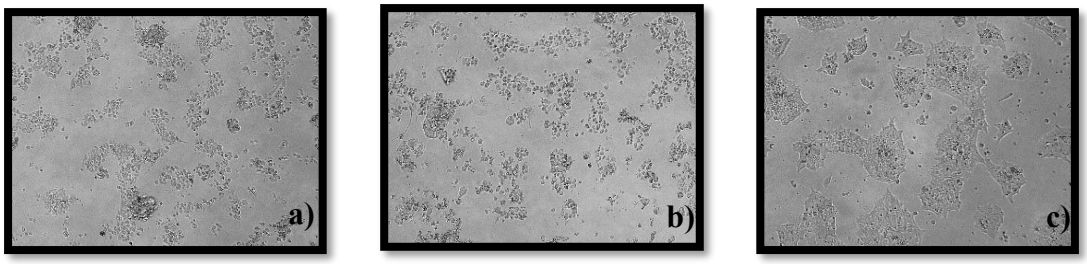


Figure: 5.43 Effect of Naphthalene on HEK Cell Line at concentration a) 500, b) 125 and c) 31.25 µg/mL

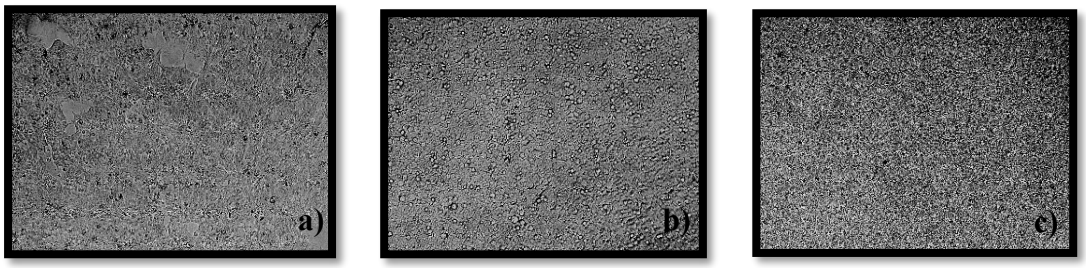


Figure: 5.44 Effect of Acenaphthene on HEK Cell Line at concentration a) 500, b) 125 and c) 31.25 µg/mL

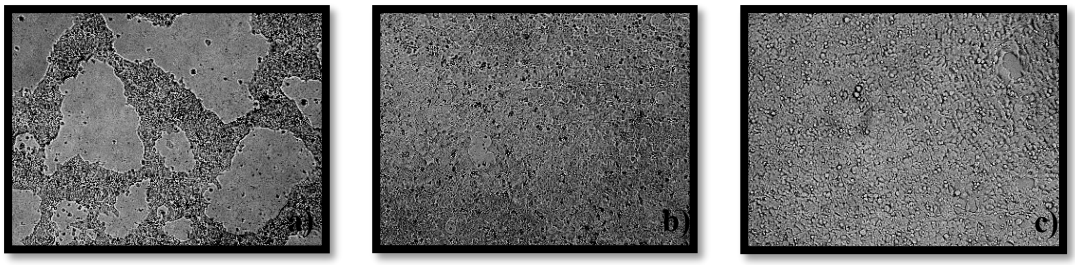


Figure: 5.45 Effect of Anthracene on HEK Cell Line at concentration a) 500, b) 125 and c) 31.25 µg/mL

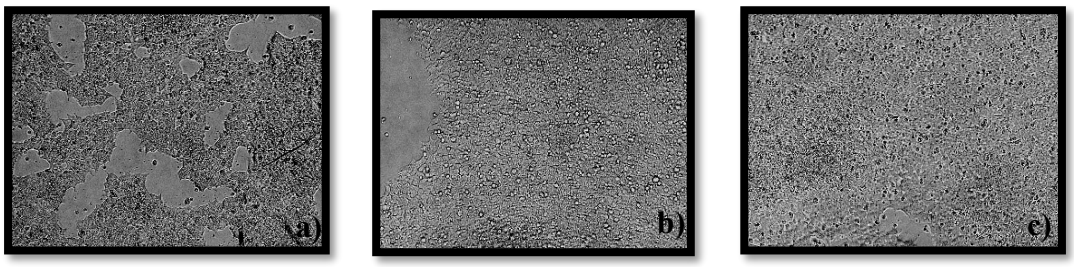


Figure: 5.46 Effect of Fluoranthene on HEK Cell Line at concentration a) 500, b) 125 and c) 31.25 µg/mL

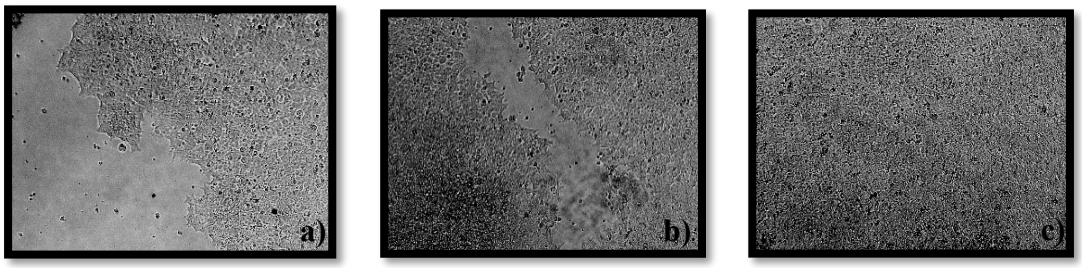


Figure: 5.47 Effect of Pyrene on HEK Cell Line at concentration a) 500, b) 125 and c) 31.25 µg/mL

Figure: 5.48 Effect of Alpha Methyl Styrene on MCF7 Cell Line at concentration a) 500, b) 62.50 and c) 15.62 µg/mL

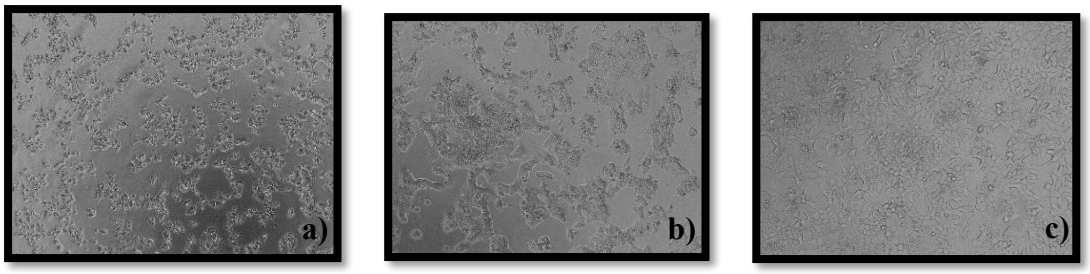


Figure: 5.49 Effect of Diethyl Azelate on MCF7 Cell Line at concentration a) 500, b) 62.50 and c) 15.62 µg/mL

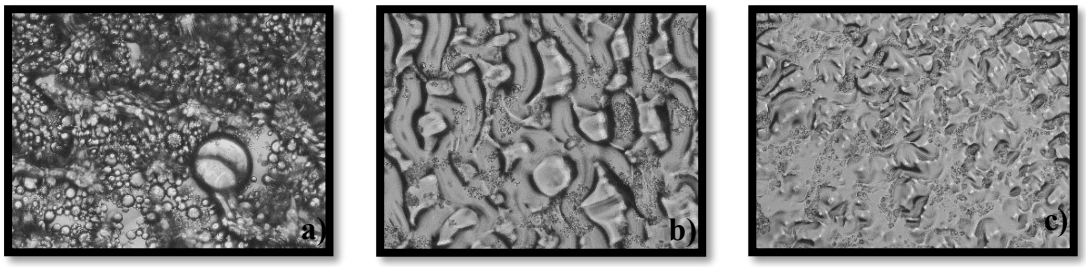
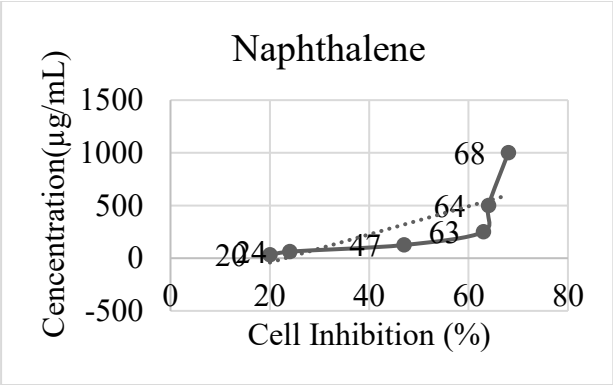


Figure: 5.50 Effect of Tributyrin on MCF7 Cell Line at concentration a) 500, b) 62.50 and c) 15.62 µg/mL

5.6.3 Quantification of Cell Inhibition by IC50

- One mechanism that regulates cell migration, growth, and proliferation is cell inhibition. It may allude to contact inhibition, a characteristic of healthy cells that stops them from proliferating when they meet one another. Cell cycle inhibitors, which are chemicals that prevent cells from dividing, are another name for cell inhibition. Signalling pathways are activated when cells in a culture reach a specific density and come into touch with one another, which prevents cell migration and division. In order to avoid excessive development and preserve tissue homeostasis, contact inhibition aids in the maintenance of a single, ordered cell layer inside a tissue.
- A lower IC50 value indicates a more potent cytotoxic effect. In a cell line MTT assay, the "IC50" value is the concentration of a tested compound that inhibits 50% of cell viability. This value basically indicates the half-maximal inhibitory concentration required to significantly affect cell growth within that cell line, as

measured by the absorbance of the formazan dye produced by metabolically active cells. IC50 determined by calculating the concentration at which cell viability is 50% reduced by graphing the percentage of cell viability against various concentrations of the tested substance.



Y= 13.34x – 307.89

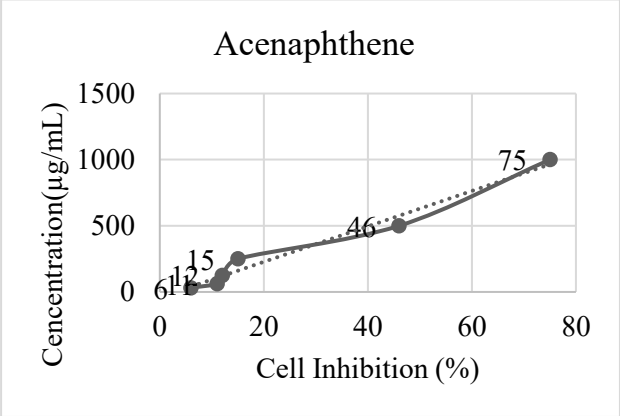
Y= 50

x= 50 + 307.89

13.34

IC50 (x)= 26.82 µg/mL

Figure: 5.51 IC50 calculation of Naphthalene



Y= 13.402x – 40.442

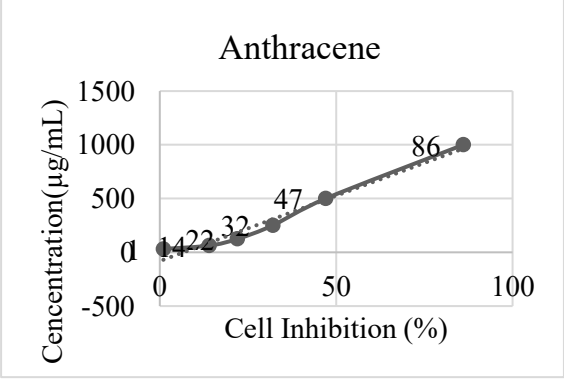
Y= 50

x= 50 + 40.44

13.40

IC50 (x)= 6.74 µg/mL

Figure: 5.52 IC50 calculation of Acenaphthene



Y= 12.169x – 81.57

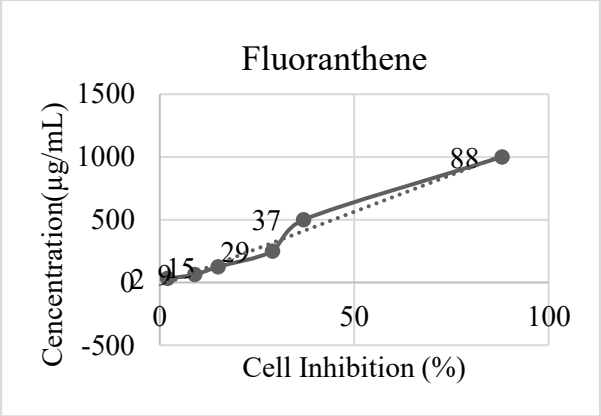
Y= 50

x= 50 + 81.57

12.16

IC50 (x)= 10.81 µg/mL

Figure: 5.53 IC50 calculation of Anthracene



Y= 11.757x – 24.594

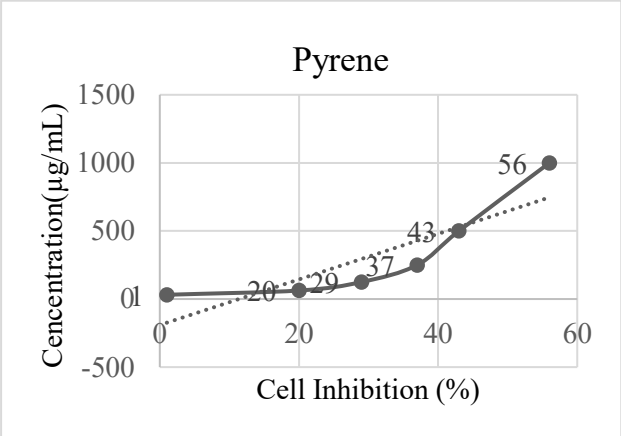
Y= 50

x= 50 + 24.59

11.75

IC50 (x)= 6.34 µg/mL

Figure: 5.54 IC50 calculation of Fluoranthene



Y= 16.735x – 190.66

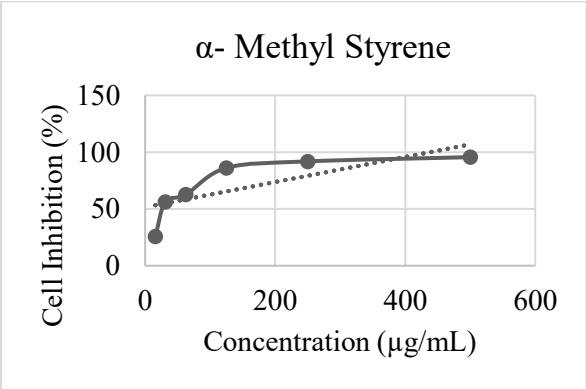
Y= 50

x= 50 + 190.66

16.73

IC50 (x)= 14.38 µg/mL

Figure: 5.55 IC50 calculation of Pyrene



Y= 0.1105x + 51.59

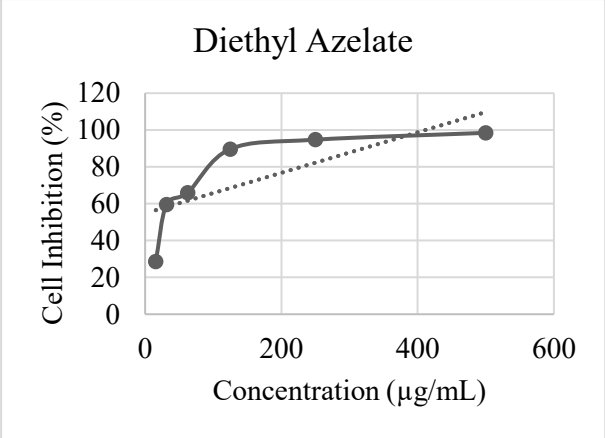
Y= 50

x= 50 - 51.59

0.1105

IC50 (x)= 14.38 µg/mL

Figure: 5.56 IC50 calculation of Alpha Methyl Styrene



Y= 0.1099x + 54.777

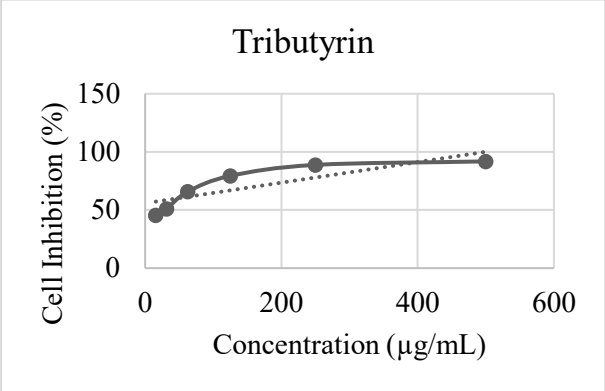
Y= 50

x= 50 – 54.777

0.1099

IC50 (x)= 43.46 µg/mL

Figure: 5.57 IC50 calculation of Diethyl Azelate



Y= 0.0884x + 55.829

Y= 50

x= 50 – 55.829

0.0884

IC50 (x)= 65.93 µg/mL

Figure: 5.58 IC50 calculation of Tributyrin

- Inclusive the results were started from the identification through FT-IR spectra. All eight analytes were identified by FT-IR and spectra showing the details about the molecules presented and matched the spectra of standard and test analytes completely. The method was developed by using gas chromatography-mass spectrometry. The parameters of the instrument were set on the basis of review of literature and trials and error methods. Optimized chromatographic conditions were adjusted. The results of mass spectra were showing the structural and molecular mass representation of all the analytes. According to the mass spectra all eight analytes were identified by the NIST17 library available in the instrument already. The method was validated according to the

ICH Q2(R1) guideline for the validation of analytical method validation. The parameters like linearity and range, accuracy, precision, robustness, detection limit and quantification limit, specificity was performed, and all the data were found within the acceptance criteria. The method was successfully applied on the pharmaceutical formulation by reflux, pH solvents and sunlight extraction techniques. Liquid pharmaceutical formulations were selected as they are prone to the leaching as specially interference of the packaging materials. Lastly the cytotoxicity study was performed to test the activity of all analytes onto the cells and for that two cell lines were adopted, amongst them one was HEK293, and another one was MCF7. At the end IC50 of the cell inhibition was calculated.

Chapter- 6

Summary and Conclusion

A rapid, selective, sensitive and stable gas chromatography–tandem mass spectrometric (GC-MS/MS) methods has been developed and fully validated according to ICH Q2 (R1) guideline for the simultaneous estimation of selected two groups of non-intentionally added substances in terms of extractables and leachables in pharmaceutical formulations.

Sample preparation involved stock solution of selected leachables standards by acetone. Analytes were separated on column SH-Rxi-5 Sil MS (30.0m, 0.25mm, 0.25µm) using helium as a carrier gas at a flow rate of 15.5 and 24.3 mL/min for both groups respectively and injection volume was adjusted at lowest i.e. 1 µl. Detection was carried out using electro spray positive ionization mass spectrometry.

The detection was performed by recording a mass of naphthalene was 128, 127 and 129; mass of acenaphthene was 153, 154 and 152; mass of anthracene was 178, 176 and 76; mass of fluoranthene was 202, 200 and 101; mass of pyrene was 202, 200 and 101; mass of alpha methyl styrene was 118, 117 and 103; mass of diethyl azelate was 83, 55 and 152; mass of tributyrin was 72, 44 and 43. Both the method was found linear as per the value of R^2 for both the group was found to be ≥ 0.990 from the range 30µg/mL to 10µg/mL for group 1 and for group 2 alpha-methyl styrene, diethyl azelate and tributyrin it was found from 250-50 ng/mL, 250-50 ng/mL and 50-10 ng/mL respectively. The retention time for group 1 i.e., naphthalene, acenaphthene, anthracene, fluoranthene and pyrene it was found to be 14.34±0.2 minutes, 21.41±0.2 minutes, 27.72±0.2 minutes, 32.43±0.2 minutes, 33.19±0.2 minutes respectively and

for group 2 i.e., alpha methyl styrene, diethyl azelate and tributyrin it was found to be 5.72±0.2 minutes, 23.77±0.2 minutes and 26.69±0.2 minutes respectively. The value for % recovery was found to be 98-102% i.e., ±2% of 100% for all standards, also the developed method was within the range of %CV i.e., ≤2%. Other parameters like robustness were performed using various parameters like change in flow rate, temperature and hold time and pressure was found to be within the range. Specificity data for both the group was found to be specific for analytes only.

Both the developed methods were applied to the pharmaceutical formulations using various extraction techniques. For the application of the developed methods reflux, pH and sunlight extraction techniques were used. The data of applications was found within the range of acceptable daily intake for all the analytes excluding naphthalene, as the quantification data of naphthalene was not within the acceptable range i.e., more than 1.5 µg/mL. For pharmaceuticals, a hypothetically 1 in 105 cancer occurrence is considered confirmed and a daily intake worth of 1.5 µg per day for an unaffected impurity is determined. (ichm7 r1, iso 10993-18:2005, 21 CFR Subpart A, Sec 600.3 (h);(h)(6)).

The toxicological study was performed by using human cell lines. For group 1 of selected analytes HEK-293 (is a cell line exhibiting epithelial morphology that was isolated from the kidney of a human embryo) cell line and for group 2 of selected analytes MCF 7 (Michigan Cancer Foundation) (Human Breast Cancer) (is a cell line derived from the pleural effusion of a 69-year-old woman with breast adenocarcinoma) cell line was used. Here, for the study of both the groups MTT assay was performed and cell inhibition i.e., IC 50 was calculated.

The following are the conclusion drawn from the study: All the selected substances samples were found reported as leachables from plastic materials. The use of plastic is increasing day by day but with the use of plastic the safety, efficacy and quality of the materials needs to be addressed. As the need of society for packaging material in terms of costing, weight, usability, availability, etc., can be majorly fulfilled by plastic material over glass or metal or any other materials. It was observed that the pharmaceutical formulations particularly parenteral and ophthalmic drug products are available in plastic as a packaging material and plastic has a major disadvantage of leaching that are not intentionally added but it came to the product throughout the whole life of product starting from its manufacturing to its use. Along with pharmaceuticals, many other products are also served with plastic packaging like food, cosmetics, etc., So, it may produce harmful effects on the patients who are the consumers of the products or the end users. According to the PQRI, such potential materials can cause serious disorders like cancer.

To address certain problems many regulatory bodies are working and have some guidelines for extractables and leachables, containers and closures, packaging materials, etc. PDA (Parenteral Drug Association) working with PQRI (Product Quality Research Institute), USFDA (United States Food and Drug Administration) has US Food, Drug and Cosmetic (USFDA) Act Section 501(a)(3) and Biologics 21 CFR 600.11(h), other regulatory bodies like EMEA (Europe, the Middle East and Africa), ICH Q3E- guideline for extractables and leachables, Indian Pharmacopoeia (IP), Schedule M of drug and cosmetics act, etc.

Chapter- 7

References

A·塔哈, J·T·费尔特斯, Polymeric substrate having a surface with reduced biomolecule adhesion and thermoplastic articles of such substrate. China Patent CN108463493B. 2021-10-15. (https://patents.google.com/patent/CN108463493B) (Accessed on 18/12/2024).

Armstrong, L., Senyurt, F., Narayan, V., Wang, X., Alquier, L., Vas, G., 2013. Stir bar sorptive extraction combined with GC-MS/MS for determination of low level leachable components from implantable medical devices. J Pharm Biomed Anal. 74:162-70.

Athenstaed, B. LOW POLLUTANT DIALYSIS SOLUTION. United States Patent US 10, 088, 457 B2. Oct. 2, 2018. (https://patents.google.com/patent/US10088457B2) (Accessed on 18/12/2024).

Benaniba, M.T. and Massardier-Nageotte, V., 2010. Evaluation effects of biobased plasticizer on the thermal, mechanical, dynamical mechanical properties, and permanence of plasticized PVC. *Journal of applied polymer science*, *118*(6), pp.3499-3508.

Bernard, M., Jubeli, E., Pungente, D., Yagoubi, N., 2018. Biocompatibility of polymer-based biomaterials and medical devices–regulations, in vitro screening and risk-management. Biomaterials science. 6(8):2025-53.

BIOTECH, S.S., 2008. Recommendations for extractables and leachables testing. *BioProcess International*.

Budde, D., Albano, L., Noll, T., Jurkiewicz, E., 2021. Interaction of leachable model compounds and their impact on Chinese hamster ovary cell cultivation. Biotechnology Progress. 37(4):e3150.

Chauhan, A., Goyal, M.K. and Chauhan, P., 2014. GC-MS technique and its analytical applications in science and technology. *J Anal Bioanal Tech*, *5*(6), p.222.

Colton, R., 2008. Extractables and Leachables Subcommittee of the Bio-Process Systems Alliance. Recommendations for Extractables and Leachables Testing, Part 1: Introduction, Regulatory Issues, and Risk Assessment. *BioProcess Int*, *6*(1), pp.44-52.

Cuadros-Rodriguez, L., Lazuen-Muros, M., Ruiz-Samblas, C., Navas-Iglesias, N., 2020. Leachables from plastic materials in contact with drugs. State of the art and review of current analytical approaches. Int J Pharm. 583:119332.

Ding, W., Madsen, G., Mahajan, E., O'Connor, S. and Wong, K., 2014. Standardized extractables testing protocol for single-use systems in biomanufacturing. *Pharmaceutical Engineering*, *34*(6), pp.1-11.

Dorival-García, N., Bones, J., 2017. Evaluation of solvent systems for optimized extractables studies of single use bioprocessing solutions. Journal of Chromatography A. 1; 1513:69-77.

Dorival-García, N., Larsson, I., Bones, J., 2017. Non-volatile extractable analysis of prefilled syringes for parenteral administration of drug products. Journal of Pharmaceutical and Biomedical Analysis. 142:337-42.

Gao, Y., Allison, N., 2016. Extractables and leachables issues with the application of single use technology in the biopharmaceutical industry. Journal of Chemical Technology & Biotechnology. 91(2):289-95.

Gollapalli, R., Singh, G., Blinder, A., Brittin, J., Sengupta, A., Mondal, B., Patel, M., Pati, B., Lee, J., Ghode, A., Kote, M., 2019. Identification of an Adduct Impurity of an Active Pharmaceutical Ingredient and a Leachable in an Ophthalmic Drug Product Using LC-QTOF. Journal of pharmaceutical sciences. 1;108(10):3187-93.

Guerra, P., Kim, M., Teslic, S., Alaee, M. and Smyth, S.A., 2015. Bisphenol-A removal in various wastewater treatment processes: operational conditions, mass balance, and optimization. *Journal of environmental manadgement*, *152*, pp.192-200.

He, G., Li, C., Zhang, T., Zhao, J., Sharma, V.K. and Cizmas, L., 2017. Transformation of bisphenol A during chloramination in a pilot-scale water distribution system: Effect of pH, flow velocity and type of pipes. *Chemical Engineering Journal*, *312*, pp.275-287.

Heim, T.H. and Dietrich, A.M., 2007. Sensory aspects and water quality impacts of chlorinated and chloraminated drinking water in contact with HDPE and cPVC pipe. *Water research*, *41*(4), pp.757-764.

Hernández-Fernández, J., Pérez-Mendoza, J., Ortega-Toro, R., 2024. Quantification of Irgafos P-168 and Degradative Profile in Samples of a Polypropylene/Polyethylene Composite Using Microwave, Ultrasound and Soxhlet Extraction Techniques. Journal of Composites Science. 8(4).

Hernández-Reyes, A., Guzmán-Albores, M., De León-Rodríguez, A., Ruíz-Valdiviezo, M., Rodríguez-Ortiz, R., Barba-de la Rosa, P., 2024. Toxicological and Sedative Effects of Chipilin (Crotalaria longirostrata) Leaf Extracts Obtained by Maceration and Supercritical Fluid Extraction. ACS Omega. 9(17):18862-71.

http://www.ipapharma.org/events/Regulatory%20Perspectives%20%20Extractables%20&%20Leachables%20Dr.%20S.pdf (Accessed on 19/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Acenaphthene (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/alpha-METHYL-STYRENE (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Anthracene (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Diethyl-azelate (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Fluoranthene (Accessed on 26/04/2019)

https://pubchem.ncbi.nlm.nih.gov/compound/Naphthalene (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Pyrene (Accessed on 26/12/2024)

https://pubchem.ncbi.nlm.nih.gov/compound/Tributyrin (Accessed on 26/12/2024)

Jenke, D. 2009. Compatibility of Pharmaceutical Products and Contact Materials: Safety Considerations Associated with Extractables and Leachables. 10.1002/9780470459416.

Jenke, D. and Carlson, T., 2014. A compilation of safety impact information for extractables associated with materials used in pharmaceutical packaging, delivery,

administration, and manufacturing systems. *PDA journal of pharmaceutical science and technology*, *68*(5), pp.407-455.

Jenke, D., Castner, J., Egert, T., Feinberg, T., Hendricker, A., Houston, C., Hunt, D.G., Lynch, M., Shaw, A., Nicholas, K. and Norwood, D.L., 2013. Extractables characterization for five materials of construction representative of packaging systems used for parenteral and ophthalmic drug products. *PDA journal of pharmaceutical science and technology*, *67*(5), pp.448-511.

Jenke, D., Jene, J., Poss, M., Story, J., Tsilipetros, T., Odufu, A., Terbush, W., 2005. Accumulation of extractables in buffer solutions from a polyolefin plastic container. International journal of pharmaceutics. 13;297(1-2):120-33.

Kapp, T., Vetter, W., 2006. Structure Elucidation of an Artifact Discharging from Rubber-Based Vial Closures by Means of Gas Chromatography/Tandem Mass Spectrometry. Analytical Chemistry. 78(23):8156-61.

Kelly, S., McSweeney, S., Coleman, O., Carillo, S., Henry, M., Chandran, D., Kellett, A., Bones, J., Clynes, M., Meleady, P., Barron, N., 2016. Process-relevant concentrations of the leachable b D t BPP impact negatively on C HO cell production characteristics. Biotechnology progress. 32(6):1547-58.

Khakimullin, N., Zakirova, Y., Khusainov, D., 2020. Determination of the Composition of Substances Migrating from Plugs Based on Bromo-Butyl Rubber into Infusion and Injection Preparations. InKey Engineering Materials. *Trans Tech Publications Ltd*. Vol. 869, pp. 135-139.

Li, K., Rogers, G., Nashed-Samuel, Y., Lee, H., Mire-Sluis, A., Cherney, B., Forster, R., Yeh, P. and Markovic, I., 2015. Creating a holistic extractables and leachables (E&L) program for biotechnology products. *PDA journal of pharmaceutical science and technology*, *69*(5), pp.590-619.

Lipke, U., Haverkamp, J.B., Zapf, T. and Lipperheide, C., 2016. Matrix effect on leaching of Bisphenol A diglycidyl ether (BADGE) from epoxy resin based inner lacquer of

aluminium tubes into semi-solid dosage forms. *European Journal of Pharmaceutics and Biopharmaceutics*, *101*, pp.1-8.

Liu, D., Nashed-Samuel, Y., Bondarenko, P.V., Brems, D.N. and Ren, D., 2012. Interactions between therapeutic proteins and acrylic acid leachable. *PDA journal of pharmaceutical science and technology*, *66*(1), pp.12-19.

Missen, A.W. and Dickson, S.J., 1974. Contamination of blood samples by plasticizer in evacuated tubes. *Clinical Chemistry*, *20*(9), pp.1247-1247.

Moly, K.A., Radusch, H.J., Androsh, R., Bhagawan, S.S. and Thomas, S., 2005. Nonisothermal crystallisation, melting behavior and wide angle X-ray scattering investigations on linear low density polyethylene (LLDPE)/ethylene vinyl acetate (EVA) blends: effects of compatibilisation and dynamic crosslinking. *European polymer journal*, *41*(6), pp.1410-1419.

Norwood, D., Fleck, L., Seyyal, E., Michelson, A., Vas, G., 2022. Evaluation of Extraction Conditions for Volatile Extractables from Polypropylene, PBT Resins, and Chlorobutyl Rubber Elastomers Using a Variety of Solvents and Extraction Techniques. Reviews in Separation Sciences. 4(1):e22003-e.

Norwood, L., Jenke, D., Manolescu, C., Pennino, S. and Grinberg, N., 2009. HPLC and LC/MS analysis of pharmaceutical container closure system leachables and extractables. *Journal of Liquid Chromatography & Related Technologies®*, *32*(11-12), pp.1768-1827.

Norwood, L., Paskiet, D., Ruberto, M., Feinberg, T., Schroeder, A., Poochikian, G., et al. 2008. Best Practices for Extractables and Leachables in Orally Inhaled and Nasal Drug Products: An Overview of the PQRI Recommendations. Pharmaceutical Research. 25(4):727-39.

Pan, C., Harmon, F., Toscano, K., Liu, F., Vivilecchia, R., 2008. Strategy for identification of leachables in packaged pharmaceutical liquid formulations. Journal of Pharmaceutical and Biomedical Analysis. 46(3):520-7.

Paskiet, D., Jenke, D., Ball, D., Houston, C., Norwood, D.L. and Markovic, I., 2013. The Product Quality Research Institute (PQRI) leachables and extractables working group initiatives for parenteral and ophthalmic drug product (PODP). *PDA journal of pharmaceutical science and technology*, *67*(5), pp.430-447.

Pharmacopoeia, B., 2013. British pharmacopoeia.

Pharmacopoeia, I., 2022. Government of India. *Ministry of health and family welfare*, *2*.

Proshad, R., Kormoker, T., Islam, S., Haque, A., Rahman, M., Mithu, M., 2018. Toxic effects of plastic on human health and environment: A consequences of health risk assessment in Bangladesh. International Journal of Health. 6(1):1-5.

Sforzini, A., Bersani, G., Stancari, A., Grossi, G., Bonoli, A. and Ceschel, G.C., 2001. Analysis of all-in-one parenteral nutrition admixtures by liquid chromatography and laser diffraction: study of stability. *Journal of pharmaceutical and biomedical analysis*, *24*(5-6), pp.1099-1109.

Sharma, N., Brahmankar, Y., Babar, D., Bhogle, T., Mirase, R., Rathod, R., et al. 2023. Systematic Approaches on Extractable and Leachable Study Designs in Pharmaceuticals and Medical Devices: A Review. Journal of Packaging Technology and Research. 7(3):127-45.

Shin, C., Lee, M., Kim, M., Kim, S., 2023. Simultaneous determination of the free and total forms of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate in human urine by gas chromatography-mass spectrometry. Analytical and Bioanalytical Chemistry. 415(26):6583-93.

Sica, P., Krivos, L., Kiehl, E., Pulliam, J., Henry, D., Baker, R., 2020. The role of mass spectrometry and related techniques in the analysis of extractable and leachable chemicals. Mass Spectrom Rev. 39(1-2):212-26.

Skudas, R., Adrian, K., Edelmann, B., Andrecht, S., Moya, Wc., Method for The Removal Of Leachables And/Or Extractables. European Patent Ep 3 215 246 B1. 07-08-2024. (Https://Patents.Google.Com/Patent/Ep3215246b1) (Accessed on 18/12/2024).

Skudas, R., Adrian, K., Edlemann, B., Andrecht, S., Moya, W., Carlisle, Activated Carbon for The Removal of Leachables And / Or Extractables. United States Patent US 10793592 B2. Oct. 6, 2020. (https://patents.google.com/patent/US10793592B2) (Accessed on 18/12/2024).

Thummar, K., Vasoya, R., Dama, B., Ladolkar, H., Raval, M., Sheth, N., 2020. Determination of Extractable and Leachable Phthalates from Pharmaceutical Packaging by LC-MS/MS. Analytical Chemistry Letters. 2;10(1):93-103.

Ulsaker, A. and Korsnes, M., 1977. Communication. Determination of cyclohexanone in intravenous solutions stored in PVC bags by gas chromatography. *Analyst*, *102*(1220), pp.882-883.

Wang, X., Zhu, X., Xu, W., Shen, J., Xu, J., Chen, L., et al., 2023. Determination of Acrylic Resin Monomers in Food Packaging Paper by Gas Chromatography – Tandem Mass Spectrometry (GC-MS/MS) with Formic Acid as a Protective Agent. Analytical Letters. 2023;56(14):2237-48.

Wong, D.M. and Firor, R.L., 2015. Analysis of Extractable/Leachable Compounds from Transdermal Patches Using GC/MSD Systems. *Agilent Technologies Application Note*.

www.ema.europa.eu (Accessed on 23/12/2024)

www.fda.gov (Accessed on 19/12/2024)

www.ich.org (Accessed on 03/12/2024)

www.pda.org (Accessed on 18/12/2024)

www.pqri.org (Accessed on 18/12/2024)

Zdravkovic, A., 2015. Solid phase extraction in tandem with GC/MS for the determination of semi-volatile organic substances extracted from pharmaceutical packaging/delivery systems via aqueous solvent systems. J Pharm Biomed Anal. 112:126-38.

Zhang, F., Chang, A., Karaisz, K., Feng, R., Cai, J., 2004. Structural identification of extractables from rubber closures used for pre-filled semisolid drug applicator by

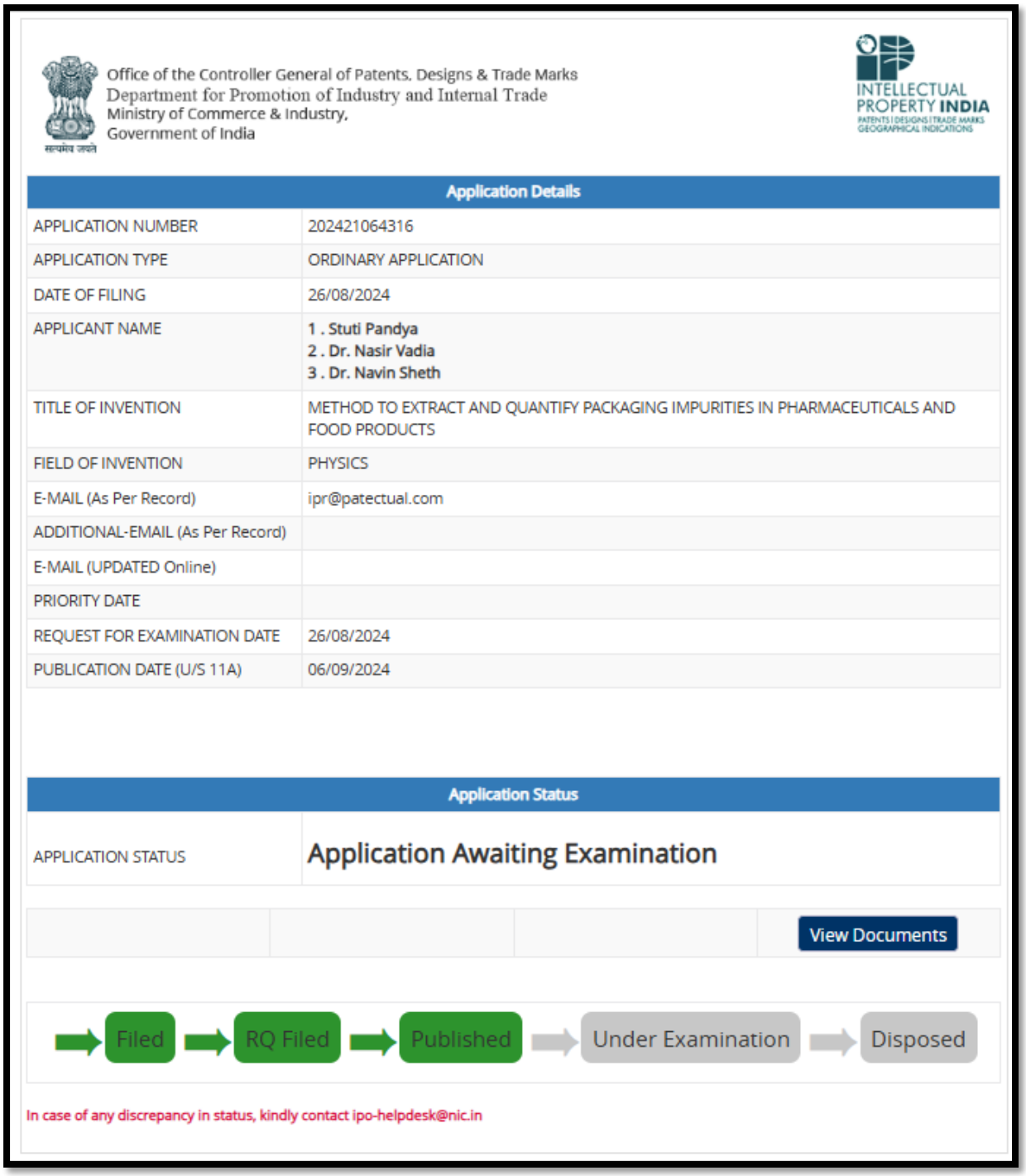
chromatography, mass spectrometry, and organic synthesis. Journal of Pharmaceutical and Biomedical Analysis. 34(5):841-9.

Zhang, Y., Sun, S., Xing, X., Du, Z., Guo, Q. and Yu, W., 2016. Detection and identification of leachables in vaccine from plastic packaging materials using UPLC-QTOF MS with self-built polymer additives library. *Analytical chemistry*, *88*(13), pp.6749-6757.

シダーグレン プロロエシエ, Dexmedetomidine premix formulation. Japan Patent JP5990832B2. 2016-09-14. (https://patents.google.com/patent/JP5990832B2) (Accessed on 18/12/2024).

Chapter 8

List of Publications



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INTELLECTUAL PROPERTY INDIA
PATENTS | DESIGNS | TRADE MARKS | GEOGRAPHICAL INDICATIONS

Application Details

APPLICATION NUMBER	202421064316
APPLICATION TYPE	ORDINARY APPLICATION
DATE OF FILING	26/08/2024
APPLICANT NAME	1 . Stuti Pandya 2 . Dr. Nasir Vadia 3 . Dr. Navin Sheth
TITLE OF INVENTION	METHOD TO EXTRACT AND QUANTIFY PACKAGING IMPURITIES IN PHARMACEUTICALS AND FOOD PRODUCTS
FIELD OF INVENTION	PHYSICS
E-MAIL (As Per Record)	ipr@patectual.com
ADDITIONAL-EMAIL (As Per Record)	
E-MAIL (UPDATED Online)	
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REQUEST FOR EXAMINATION DATE	26/08/2024
PUBLICATION DATE (U/S 11A)	06/09/2024

Application Status

APPLICATION STATUS	Application Awaiting Examination

View Documents

Filed → RQ Filed → Published → Under Examination → Disposed

In case of any discrepancy in status, kindly contact ipo-helpdesk@nic.in

Fig 8.1: Patent Filling Details

Stuti Pandya/Afr.J.Bio.Sc. 6(6) (2024) 8955-8974 ISSN: 2663-2187

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Research Paper Open Access

Categorization, Computation and Examination of Extractables and Leachables From Pharmaceuticals and Food Packaging Materials

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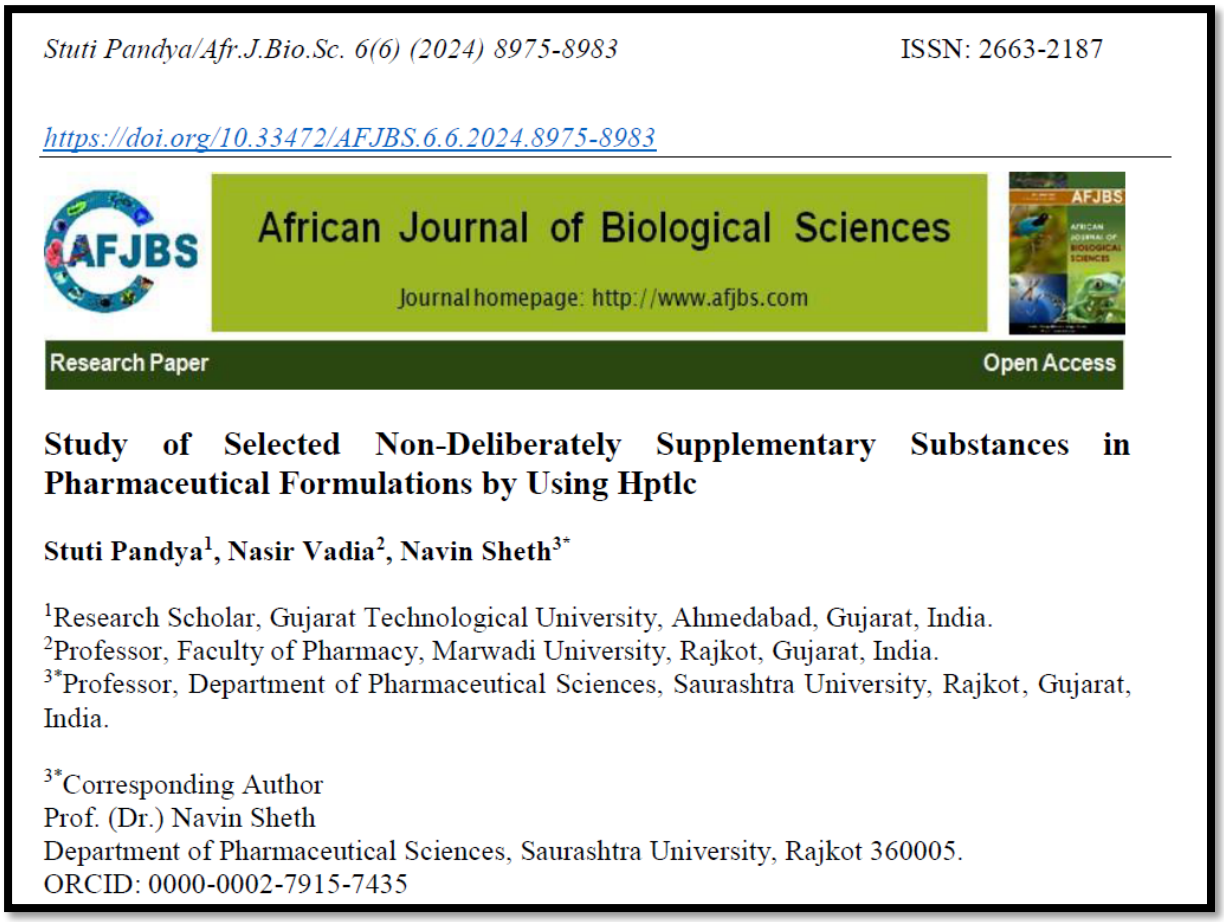
Fig 8.2: Paper Publication 1



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Research Paper Open Access

Study of Selected Non-Deliberately Supplementary Substances in Pharmaceutical Formulations by Using Hptlc

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Fig 8.3: Paper Publication 2